9-2012

Determination and Speciation of Arsenic in Environmental and Biological Samples

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DETERMINATION AND SPECIATION OF ARSENIC IN ENVIRONMENTAL AND BIOLOGICAL SAMPLES

A Dissertation Presented

By

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Submitted to the Graduate School of the

University of Massachusetts Amherst in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2012

Department of Chemistry

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DEDICATION

To my fiancée and my family

ACKNOWLEDGMENTS

Firstly, I would like to express my genuine appreciation to my advisor, Julian F. Tyson, for countless hours of advice, encouragement, and guidance throughout my graduate studies, related to my research, my graduate career, as well as in personal matters. I would also like to extend sincere gratitude to Peter Uden for his mentorship and advice. Dr. Richard Vachet and Dr. Om Parkash, members of my doctoral committee, as well as Dr. Edward Voigtman have also been very helpful in developing my critical thinking skills with their discussion points.

I would like to express gratitude to the other Tyson group members, specifically Monique Johnson, Lindsay Harris, Chengbei Li, Nan Wang, and former group members James Kearns and Prince Amoako for their camaraderie. I would also like to thank my chemistry colleagues in the entering class in 2007 for all of their support and countless hours spent in the CRC, getting through our first year of graduate school.

I would like to thank Mort Sternheim and all of the members of the STEM DIGITAL project, for providing me with the experience of presenting and engaging in research with a wider audience. I would also like to thank the members of the Environmental Analysis Laboratory, for extending my knowledge of analytical chemistry to areas outside of my own research. I would like extend a deep appreciation to my former colleagues at the Schering-Plough Research Institute, including my PIs Dr. Evan Bekos and Dr. Jennifer Vance, and Dr. Doug Richardson and Dr. Jennifer Albaneze-Walker, for their training and advisement.

Finally, I would like to express sincere gratitude to my parents, James and Lesley Berg, my sister Brittany and my best friend Kara Tremblay for their continued support, encouragement and love. I would like to especially thank my fiancée, Andres Hernandez, who has been very motivating, patient and caring throughout the years. Their support has strengthened my resolve; without it, I would not have accomplished so much.

ABSTRACT

DETERMINATION AND SPECIATION OF ARSENIC IN ENVIRONMENTAL AND BIOLOGICAL SAMPLES

SEPTEMBER 2012

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A method was developed for the determination of total arsenic in rice grain by microwave-assisted digestion inductively coupled plasma mass spectrometry. Standard calibration solutions were matrix-matched with respect to acid concentration and carbon content post-digest. The importance of eliminating the drying step during sample preparation procedures was investigated. The method was validated with spikes containing standard arsenate solutions into the rice matrix, and with certified reference material SRM1568a (rice flour) from NIST. The method was successfully applied to a commercially available rice sample.

Four arsenic species [arsenate (As(V)), arsenite (As(III)), dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA)] were extracted from rice grains by microwave-assisted extraction and separated with high performance liquid chromatography inductively coupled plasma mass spectrometry. The method includes a novel sample clean-up step involving a dialysis procedure to decrease the amount of large starch molecules in the injection solution, in order to minimize poor resolution of

chromatographic peaks and maximize column life. The method was validated with spikes of standard arsenic solutions, added to the rice matrix before the extraction procedure. Literature reference values for arsenic species quantification in SRM1568a (rice flour) were also compared. This method was successfully applied to a commercially available rice sample.

A study into improvements in reverse phase-HPLC separations of arsenic species was conducted. For the first time, a Sunfire C8 column from Waters (Milford, CT) was employed for the separation of arsenic species in rice extracts. This column was compared to a Symmetry C8 column with respect to total elution time, detection limits, interference effects, and column life, and evaluated with respect to peak resolution, shifts in retention times, and peak symmetry.

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INTRODUCTION

1.1 Arsenic

Arsenic is an element of great interest in the world of environmental science today. It is present naturally in the Earth's crust, particularly in volcanic rocks and other minerals. Arsenic ranks somewhere between 46th and 54th in the earth's crust,[2,3] is about 24th - 28th in seawater[2-4] and is about 31st in the human body.[4] Arsenic compounds are also artificially introduced to ground water and soils through various other means such as pesticides,[5] wood preservatives,[6] mining and metal-processing plant areas,[7-9] and chemical warfare agents.[10] Arsenic compounds also exist in several food additives and veterinary drugs.[11]

1.1.1 Chemical Forms of Arsenic

The chemical forms of arsenic can be found in Appendix A[12]; they include a wide range of compounds, from inorganic and organic forms, to arsenosugars and glutathione complexes. For the evaluation of arsenic in rice grain, four arsenic species are investigated most often; they are arsenite [As(III)], arsenate [As(V)], dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA). Recently, both trimethylarsine oxide (TMAO) and tetramethylarsonium ions have been identified in rice grains as well.

Another group of arsenic compounds has increasing popularity among plant studies as well. These compounds contain glutathione or phytochelatin donors.

Phytochelatins (PCs) are cysteine-containing peptides formed by plants to help detoxify essential and nonessential amounts of copper, zinc, arsenic, cadmium or mercury ions. The structure of PCs follows the formula $(\gamma\text{-Glu–Cys})_n$–Gly (n=2–11). Glutamic acid (Glu), β-alanine (Ala), serine (Ser), or a hydroxyl group from the iso-PCs can replace the glycine (Gly) groups.[13] Examples of PC structures are given in **Figure 1.1**.

1.1.2 Sources of Arsenic

There are several theories about how arsenic enters the water supply. According to Roychowdhury et al.,[14] arsenic release in aquifers is due to arsenic-rich iron pyrite in Bengal delta sediments. The author suggested that there is a link between arsenic and iron pyrite. Pyrite oxidation and iron oxyhydroxide-reduction are two suspects in the source of contaminated groundwater.

The second suggestion links arsenic uptake with microbially-mediated iron reduction.[15] Microbes reduce iron compounds present in sediment. Minerals containing arsenic and iron, act as electron donors that facilitate microbial growth. As a result, dissolution of the arsenic compounds occurs. This release results in bioavailability of the arsenic.

Arsenic release may also result from processes independent of Fe (III) minerals.[16] Research suggests that arsenic-reducing bacteria directly dissolve arsenic compounds, exploiting arsenic as an electron acceptor. These three theories dominate current studies of the mechanism of arsenic dissolution and bioavailability.

Once the arsenic becomes bioavailable, plants take up the compounds. The compounds undergo further transformations influenced by biological pathways, in an attempt to detoxify the arsenic. These pathways differ depending on the plant; therefore, the arsenic species within different plants are both numerous and diverse.[17] One way plants detoxify arsenic was suggested by Pikering et al.,[18] namely that arsenate entered the roots of Indian Mustard (*Brassica juncea*) as a phosphate analog where it was reduced to arsenite. Little arsenic traveled to the plant tissues above-ground. According to Dhankher et al.,[19] plants may trap the arsenite in the roots of the plant to prevent damage to the above-ground reproductive tissues. It can be seen, therefore, that the transformation of arsenic species in plant tissues needs to be closely monitored. The arsenic compounds should be identified and quantified in various plant and food materials in order to accurately determine the toxicity related to the arsenic.

1.1.3 Arsenic in Environmental and Biological Samples

The most common matrix examined for arsenic content is drinking water. Contamination exists in both surface water and well water. Although drinking water has usually been of first concern when looking at arsenic poisoning, recent studies show that irrigation with the contaminated water is leading to arsenic compounds being present in food crops, mostly rice and vegetables.[14,20-22] When arsenic contaminates these vital food sources, arsenic poisoning becomes a larger problem not only affecting drinking water, but also contaminating main food staples. Food stuffs such as rice, potatoes, and onions have been analyzed for their arsenic content[14,20]. Many of the potentially contaminated substances are products available for human consumption; therefore, the samples should be carefully analyzed to ensure that this human consumption does not lead to any toxic effects.

Plants are often evaluated in sections – roots, shoots, leaves, and edible parts. Dahal et al.[20] monitored arsenic uptake in plants (rice, potato, cauliflower, onion and brinjal) that were irrigated with contaminated water. The group found that arsenic content was greatest in the plant roots, followed by the shoots, leaves and finally, the edible parts. The edible sections of the plants can be further divided if the products for human consumption undergo milling processes. For example, in one study,[23] brown rice contained a significantly higher amount of arsenic than white rice. This was attributed to the fact that brown rice retains its outer layers, while in white rice those layers are removed by the milling process.

The edible sections (skin, husk and grain) of the plants are examined closely in relation to cooking strategies as well. Cooked food and the skin of the vegetables[14] were also found to contain relatively high concentrations of arsenic. This was due to the fact that the edible rice grain contained 95% of the arsenic present in the rice plant, and that cooked rice retained 75% more water. Therefore, arsenic concentrations in cooked rice samples were 2.1 times higher than in the raw sample.

1.1.4 Arsenic Toxicity

When the compounds enter the drinking water supply, they can cause significant health problems, including skin lesions and cancers of the skin, lungs, liver, kidney, colon, bladder, and prostate to exposed individuals.[24-27] The International Agency for Research on Cancer (IARC)[27] places arsenic and inorganic arsenic compounds in Group 1, which means that they are carcinogenic to humans. Dimethylarsinic acid and monomethylarsonic acid are considered possibly carcinogenic to humans and are placed in Group 2B by the IARC.

As stated in the IARC monograph,[27] arsenic enters the body and is transported through the body bond to SH groups in proteins. Arsenic can also bind to low molecular weight compounds like glutathione (GSH) and cysteine. This can alter protein binding sites and disrupt protein conformations. As(III) also has an affinity for lipoic acid and dimercaptosuccinic acid. In this way, arsenic can inhibit lipoic acid and the citric acid cycle. Arsenic can also limit mitochondrial respiration and ATP synthesis, causing damage to the DNA backbone.

Although many arsenic compounds are labeled as carcinogenic, the real toxicity of certain substances is not fully understood. This is partly due to the difficulty in speciating very low concentrations of arsenic compounds in food stuffs.[27] In order to better understand the effects that arsenic has in the body, one must first understand the arsenic species present in the water and food supplies.

1.1.5 Regulation of Arsenic Compounds

According to the World Health Organization (WHO)[28], the most important route of exposure for arsenic poisoning is through the consumption of food and beverages. The organization has set a 10 µg L^{-1} arsenic threshold value, decided upon according to the practical quantification limits and practical difficulties in removing toxic arsenic compounds from contaminated water. Any amount of arsenic above 10 µg L^{-1} in water is considered unsafe to consume.

The European Food Safety Authority (EFSA) also stated,[29] "in order to refine risk assessment of inorganic arsenic there is a need to produce speciation data for different food commodities to support dietary exposure assessment and dose-response data for the possible health effects." In an effort to address arsenic exposure concerns in the food supply, in 2009,[29] the Contaminants in the Food Chain (CONTAM) Panel of the EFSA recommended that dietary exposure to inorganic arsenic, expressed as $BMDL_{01}$ values, should be reduced to a concentration range of 0.3 to 8 μg kg^{-1} body weight per day for inorganic arsenic species.

1.2 Arsenic in Rice

Rice is one of the most widespread, main food staples in the world. The United States Department of Agriculture[30] estimates 434.3 million tons of milled-based rice was produced in 2008/2009; among the 4 largest rice-producing countries are China, India, Indonesia and Bangladesh. According to Zavala et al.[23] the global "normal" range of arsenic in rice grain is 80 to 120 μg kg^{-1}. Zhu et al.[7] claims a normal level of 150 μg kg^{-1}, and found arsenic concentrations as high as 624 μg kg^{-1} in rice grains grown in mine-impacted areas.

A recent review[31] stated that food regulations for arsenic in rice would have the most impact on reducing arsenic contamination in food stuffs. One study[32] found that the risk of exposure to arsenic is due to the consumption of rice more than drinking water; there is a 37.6% contribution from consuming rice to the maximum tolerable daily intake of arsenic compared to only 1.5% contribution from drinking water.

1.3 Determination of Total Arsenic

The difficulties of determining accurately the total arsenic concentration in rice are well illustrated in the report, by de la Calle et al.[33] of the results of a proficiency test organized by the European Union Reference Laboratory for Heavy Metals in Feed and Food with the support of the International Measurement Evaluation Program for which results from the participating laboratories were submitted by January 15th, 2010. The test material was cryogenically milled to a particle size of less than 250 µm and shown to be both (a) homogenous with respect to arsenic content at the 500 mg sample size and (b) stable during the period of the study. The assigned value for the total arsenic was determined based on the results of 7 expert laboratories each of which used their own method. The values obtained were, 139, 164, 171, 172, 176, 190, and 190 µg kg^{-1}. The range is 51, the mean is 172, and the standard deviation is 17 µg kg-1. From these data and the uncertainty associated with homogeneity and stability, an expanded uncertainty of ±18 µg kg^{-1} was calculated and thus the reference interval was 154 to 190 µg kg^{-1} (based on a coverage factor of 2). As this corresponds to approximately a 95% confidence interval, it might be expected that on the basis of chance alone, 5 of the 98 participating laboratories would submit results that fell outside this range if the laboratories methods were all equally accurate and precise. In fact, only 35 laboratories submitted results that fell within this reference interval. The organizer of the trial defined a wider target interval based on twice the "maximum acceptable standard uncertainty", of 15% of the assigned value. This 15% number was chosen on the basis of "feedback from experts, on the state of the art, and on discussions among the members of the advisory board of the proficiency trial." This target interval of 120 – 224 µg kg-1 was missed by

26 of the participants (16 of whom were low). Interestingly enough, participants were instructed to correct their results (a) for recovery and (b) for water content. Detailed instructions were provided regarding the moisture content, but no guidance was given about correcting for recovery. As it turned out, 67 participants did not correct for recovery, but only 9 did not correct for water content, which ranged from 0.5 to 14% (based on the results of the labs that did apply a correction). In the light of this variation in moisture content, it is interesting that "control laboratories are requested by the European legislation to report their results on the samples as received (not on a dry mass basis)." It would also be interesting, in the light of the findings of the loss of arsenic on "drying," which is located in section 2.4.1, to know which laboratories performed the analysis on the dried material as opposed to analyzing the "wet" (as received) material with determination of the moisture content from an experiment with a separate sample.

1.3.1 Quantification Methods for Total Arsenic

A comparison of current analytical methods for the determination of total arsenic is presented in **Table 1.1**. A variety of sample matrices have been investigated with the use of numerous types of detection instrumentation. Methods with ICP-MS detection result in a wide dynamic range compared with other methods such as hydride generation-atomic fluorescence spectrometry (HG-AFS) and hydride generation- atomic absorption spectrometry (HG-AAS). The limits of detection for current methods are in the ppb range, and continue to move toward ppt levels.

1.3.2 Sample Preparation

There are discrepancies in the literature with regards to drying and digestion procedures. Plant samples are traditionally dried in an oven to deplete the moisture content.[41] Raab et al.[42] dried rice grains in an oven at 80 °C until constant weight was reached. Baba et al.[10] determined arsenic on the basis of wet weight, then oven dried the samples at 105 °C to obtain the moisture content. In the IMEP study,[33] participating laboratories were given specific instructions on how to calculate the moisture content of the sample. Samples were analyzed as received, and results were reported as dry mass based on a moisture correction factor.

Sample size is also a concern when considering the appropriate weight for a homogeneous, accurate representation of the bulk material. The certificate for SRM 1568a suggests that a minimum of 500 mg be analyzed. Zhu et al.[7] made determinations based on a 0.1 – 0.2 g of powdered grains, while Zavala et al.[23] used as much as 9 g of rice. There is a clear discrepancy here. Sample size should be chosen carefully, with great attention paid to the accurate reproducibility of the results based on sample size.

1.3.3 Digestion

For total arsenic determination in rice, popular digestion techniques make use of digestion blocks[7,43] or microwave-assisted digestions.[44-47] Microwave digestion techniques can achieve complete digestion of rice materials. The advantages of microwave digestion techniques are as follows:[48] (1) performing digestions in a closed vessel can achieve digestion in less time because the high pressure in the vessels raises the boiling point of the acid involved, (2) sources of contamination are minimized in a closed vessel system - there is no need to add acid continuously throughout the digestion process and no airborne contaminants enter the vessels during the process (3) the entire system heats uniformly, and (4) elements that can form volatile intermediates can be digested without loss.

Techniques often involve acid digestions, involving nitric acid alone,[7,49] a combination of nitric acid and hydrogen peroxide,[23,50-51] the use of hydrofluoric,[47] or perchloric acids.[52-53] Enzymatic digestions with amylase and protease have also been employed to extract arsenic compounds out of the solid sample matrix and into solution form. Inconsistencies in methods are also realized with a variety of temperature programs, ramping to final temperatures of 80 °C,[53] 120 °C,[7] 145 °C,[23] 180 °C,[10] and 210 °C.[45] The appropriate digestion method must be investigated with respect to these parameters.

1.3.4 Detection by ICP-MS

Offline coupling of microwave digestion with ICP-MS has its advantages. ICP-MS is a highly sensitive technique that is widely used for trace elemental detection in aqueous sample matrices. However, other matrix components can result in artificially high or low results for a given element. For arsenic determination in rice, analysis by ICP-MS requires strict attention to matrix considerations such as acid concentration,[54] chloride interference at m/z 75 and carbon-loading effects.[55]

Varying acid concentrations affect local plasma temperatures within the sampling volume, which affects ion kinetic energies and transmission efficiency from the plasma to the MS detector. Increasing the acid concentration decreases plasma temperatures, which, in turn, lowers the ionization efficiency of a given analyte. Nitric acid, in particular, has been studied with respect to its evaporation from the water droplets surface, the relative contribution of bond-breaking to the overall energy of the system, and the contribution of the decomposition products to the efficient transfer of energy to the central channel of the plasma.

The effect of chloride concentration on arsenic signal response is well documented. The argon-chloride dimer ($[^{40}Ar^{35}Cl]^+$) arises from the argon carrier gas in the plasma, and high chloride concentrations in the sample matrix. The dimer causes an isobaric interference at m/z 75, which potentially results in artificially high signal response values.

Signal response values can also become artificially high with the introduction of carbon to the plasma. Carbon is known to enhance the ionization intensity of elements in the plasma in order of decreasing mass. Although the exact mechanism is unknown, several studies have proposed that there is a charge transfer from C^+-species to the analyte atom, resulting in an increase signal response for the analyte.

1.4 Speciation of Arsenic

1.4.1 Separation Techniques

High performance liquid chromatography (HPLC), reversed phase high performance liquid chromatography (RPLC), gas chromatography (GC), and flow injection are commonly used separation techniques involved in the evaluation of arsenic species. More recently, x-ray absorption near edge spectroscopy (XANES) and synchrotron XRF microscopy have generated relative quantification of arsenic species in rice grain samples.

1.4.1.1 Normal Phase HPLC

In a review of current chromatographic, elemental speciation techniques coupled to ICP-MS,[56] several improvements in separations were noted. When performing HPLC, minimizing the transfer line length and internal diameter reduces peak broadening. Several different nebulizers can also be used when sample matrix issues become a problem. Organic phases should appear in low concentrations in order to reduce plasma instability.

Separation of As(III), As(V), MMA and DMA from spiked soils and standard reference materials[35] was determined by HPLC-ICP-MS. A Hamilton PRP X-100, 10 µm quaternary amine anion exchange column was used with 10 mM ammonium dihydrogen phosphate as the mobile phase. In another HPLC procedure,[36] a mobile phase of mainly methyl isobutyl ketone (MIBK) and another phase of mainly toluene further separated and helped to identify ten different arsenolipids in fish oils.

The investigation of the terrestrial plant,[57] *Ceratophyllum demersum*, was achieved by an anion-exchange PRP-X100 column and a cation-exchange Zobax 300-SCX column. Arsenic compounds in rice samples[58] were separated by a Dionex AS7 anion-exchange column. A gradient elution with dilute nitric acid was employed.

Arsenic speciation in rice[59] was also achieved with a PRP-X100 anion-exchange column at pH 2.8 in 4 mM pyridine formate.

1.4.1.2 Reversed Phase HPLC

A sequential injection dual mini-column system[60] coupled to hydride generation atomic fluorescence spectrometry has allowed for the separation of inorganic As(III) and As(V). Both a C18 column and a 717 anion-exchange resin column were used. The C18 column affectively retains a complex of As(III) and APDC, and the sorption of As(V)is achieved with the 717 anion exchange resin.

A gradient method[61] using an AS16 anion exchange column separated As(III), As(V), DMA, MMA. Arsenobetaine coeluted with other organic forms, and could therefore not be accurately quantified. In some cases, RPLC can minimize the co-elution of some species with arsenobetaine.[56]

A C18- RPLC column separated the following six arsenic-containing compounds in a human urine sample:[38] arsenocholine (ASC), arsenobetaine (ASB), dimethylarsonic acid (DMA), methylarsonic acid (MMA), arsenite and arsenate. Tetrabutylammonium hydroxide ion pair reagent was used as the mobile phase in the column. Methanol was also used at a pH of 5.7-5.8, adjusted with malonic acid. The sample was analyzed by ICP-MS at m/z 75.

After extraction from sunflowers,[62] arsenic phytochelatin complexes were separated by a C18 PRLC column with a mobile phase consisting of 1% formic acid in water and a 0 – 13% methanol gradient. A flow rate of 1 mL minute^{-1} was used.

1.4.1.3 Gas Chromatography

Inorganic arsenic, DMA and MMA were determined in seawater, wine, beer and infant food[34] by GC-AED. Analytes were separated on a HP-5, 5% diphenyl 95% dimethyl polysiloxane capillary column. The concentrations of inorganic arsenic in seawater and wine samples ranged from 1-40 ng mL^{-1}. DMA in infant food ranged from 20-80 ng g^{-1}.

1.4.1.4 X-ray Techniques

X-ray techniques have been employed to determine arsenic speciation in rice grain. Bluemlein et al.[13] characterized arsenic compounds in plants by X-ray absorption near-edge spectroscopy (XANES). The results of that study suggested that over 50% of arsenic in the plant *Thunbergia alata* is bound to sulfur peptides in phytochelatin complexes, mainly PC2, PC3 and PC4. Meharg et al.[63] applied XANES to locate arsenic in polished and unpolished rice grains. The studies identified inorganic forms of arsenic as well as DMA in the whole grain material. Pickering et al.[18] employed XANES to identify As(III)-dimercaptosuccinate and As(III)-*tris*-glutathione in the roots and shoots of the Indian mustard plant. Although XANES spectra have been used to identify arsenic species and their location in rice grain material, there are several drawbacks to the technique. As stated in a recent review,[64] data from the spectra can only identify species that are present at 5-10% of the total arsenic in the sample, because the spectra are the result of a weighted sum. Photoreduction of the sample during analysis should also be monitored.[64]

Synchrotron X-ray fluorescence (XRF) microscopy has also been employed[65] in arsenic speciation studies. The researchers pointed out that absolute quantification with this technique was difficult for two reasons. First, the accuracy of the method relies on the availability of matrix-matched standards that are homogeneous on the micron scale. The reliability of the method is also dependent on uniformity with respect to the thickness of the sample. As stated in a recent review,[64] uneven thickness or density of the sample could lead to false hotspots in the spectra.

1.4.2 Extraction Methods

Generally, extraction procedures should always keep the species intact, and one must always attempt to recover all of the arsenic species present. The extraction technique should be developed with a specific matrix in mind.

There is no universally applied extraction procedure for arsenic compounds. Dilute acids, such as nitric acid[66] and trifluoroacetic acid[63] have been used to extract arsenic species from rice grain. Although the extraction efficiencies are acceptable for these acids, often it becomes impossible to distinguish between the inorganic forms of arsenic [As(III) and As(V)]; values for these species are often combined and reported as “inorganic” arsenic species. One study,[66] reported using 0.28 M nitric acid as the extractant, stating that inorganic arsenic species transformation was not observed. The authors claimed that the mild oxidation conditions of the dilute acid are balanced by the reduction capabilities of thiolate compounds in the rice grain matrix.

Enzymes, such as protease XIV and α-amylase[67] have also been used as extractants for arsenic speciation in rice grain. Protease XIV reagent is known to have an arsenate contamination.[67] Therefore, results obtained using this enzyme for extracting arsenic species must be carefully examined.

Methanol/water extractions have also been reported.[68] Samples are usually extracted with a 1:1 ratio of methanol to water. High concentrations of organic carbon materials can affect plasma stability, and in lower amounts can improve the sensitivity of the arsenic signal response. Special care must be taken to ensure that the methanol in the sample does not lead to inaccuracy of the results and an overestimation of arsenic species present in the sample.

Hot water extractions have been investigated as well. Narukawa et al.[43] extracted arsenic species from rice grain using a microwave-assisted water extraction. Rice grain samples in water were heated to 80 ºC for 30 minutes. The resulting extract was centrifuged and filtered before injection onto an ODS-L column.

Dietz et al.[69] performed ultrasound probe sonication (UPS) to assist their digestion methods. Standing wave patterns create non-uniformity with respect to density, which in turn creates areas of extreme localized high temperatures and pressures. Through ultrasonic probe sonication, the solid is partially extracted into the solvent.[69] Ultrasound probe sonication was performed in combination with enzymatic digestion on rice grain and straw samples.[70] According Sanz,[70] UPS techniques have often neglected to deal with the insoluble fraction of the sample. Sample sizes of 0.3 g of rice grain or 0.1 g of rice straw were placed together with 10 mg of α-amylase and 3 mL of water. Sonication was applied to the mixture for 60 s. Next, 30 mg of protease was added and sonication continued for 120 s. Following separation by HPLC, the arsenic species were quantified by -ICP-MS.

Chen et al.[60] investigated carbon nanofibers as a solid phase extraction technique for separation of inorganic arsenic species. With ammonium pyrrolidinedithiocarbamate (APDC) present at a pH between 1.0 and 3.0, As(III) was retained in the column, while As(V) was not retained.

1.4.3 Sample Clean-up Procedures for HPLC

Organic matter present in the sample matrix can result in some unwanted column effects, such as loss of resolution between the chromatographic peaks.[71] Larger carbon complexes such as starches can also cause damage to the chromatographic column by shortening the overall column life or by creating a blockage inside the column. Rice grain samples contain significant amounts of amylose and amylopectin starches that can pose problems in HPLC analysis.

Narukawa and Chiba[72] reported that the large amount of starch materials found in polished white rice, in particular, lead to a decrease in peak resolution and degradation of their chromatographic column. The researchers also mentioned that the high viscosity of the extract solutions led to an increase in peak width and a decrease in peak height. In this case, an internal standard of arsenobetaine was used to correct for the effect.

Yuan et al.[71] reported similar undesirable effects related to both column degradation and poor peak resolution. For these reasons, they studied sample clean-up procedures[71] to reduce carbon concentrations. These techniques included running the sample through C18, activated carbon and hexane, prior to injection onto the column. It is important to investigate sample clean-up procedures in order to ensure that the chromatographic separation is not compromised and that the column life is extended to its greatest capacity.

1.4.4 Validation of speciation methods

Validation of analytical method is not widely discussed. In the case of arsenic speciation, one should reliably determine and quantify the arsenic species present, and also ensure that the method does not cause interconversion of arsenic species. **Table 1.2** highlights some of the validation procedures used in current analytical methods for the speciation of arsenic in rice.

When examining the use of spike recoveries, many discrepancies arise. In many cases, standard arsenic solutions were not spiked into the sample matrix. Researchers validated their methods by analyzing either mixed or individual standard solutions. In the presence of the sample matrix, the analyte species of interest have the potential to behave quite differently. Analyzing aqueous standard solutions is not a sufficient procedure for validating a method, as the influence of matrix side reactions is ignored. Similarly, researchers have also claimed to validate their methods by spiking standard arsenic solutions into the extract solution. The matrix effects of the rice extracts are also ignored in this case. Although the influence of the extractant may be known, once again, side reactions that may be created by compounds present in the rice sample itself are ignored. The method cannot be properly validated if these matrix effects are ignored. Standard arsenic solutions should be spiked into real samples, and carried through the entire procedure in order to determine if any species interconversion is occurring.

The use of certified reference materials can be helpful, but there is no CRM available that is certified for each of the arsenic species. Certified reference materials (SRM1568a, GWB 10010, GWB 080001, and NIES no. 10) are only certified for total arsenic. In an attempt to validate their methods, many researchers compare their speciation results to other values reported in the literature. **Table 1.3** is a table comparing literature values for SRM1568a; it was provided by Batista et al.[73] This table illustrates the variability of the speciation results for the SRM; As(III) concentrations range from 52 to 129.2 ng g^{-1}, As(V) concentrations range from 12 to 53.7 ng g^{-1}, DMA concentrations range from 31.5 to 180 ng g^{-1}, MMA concentrations range from 2 to 14.9 ng g^{-1}, and total recovery values range from 80% to 99.4%. These numbers vary greatly, suggesting that consideration only of the literature comparisons in order to validate speciation methods for arsenic in rice is not an adequate validation procedure.

It is also important to note that many extraction methods involve dilute acids, such as TFA and nitric acid. These acids make it impossible to distinguish between As(III) and As(V) because As(III) is oxidized to As(V); the results are therefore only reported as “inorganic arsenic”. Other speciation methods involve a drying step prior to analysis. Some arsenic species can be lost during this step, making complete extraction of arsenic species difficult.

Although there any many methods reported in the literature for speciation of arsenic in rice grains, it is always important to examine how each method is validated. Certified reference materials should be used when available, but many times, appropriate speciation data is not provided. Solution spikes into the matrix of interest should also be evaluated through the entire procedure.

1.5 Compound-dependent Responses

There is evidence of compound-dependent responses when quantifying arsenic species with ICP-MS. It is well known that when performing hydride generation, compounds form hydrides at different rates; therefore, compound dependency, when employing hydride generation techniques, does exist. On the other hand, compound dependent responses with plasma instrumentation are far less well understood. Pan et al.[38] noted compound dependent responses for five arsenic species. Average response factors relative to As(V) were listed for the following compounds in the mobile phase and in urine respectively: arsenocholine (0.669, 0.783), arsenobetaine (1.25, 1.32), arsenite (1.02, 1.04), DMA (1.12, 1.20), MMA (0.955, 1.02) and arsenate (1.00, 1.02). Clearly, the instrumentation responded differently depending on the chemical form of arsenic present. However, the researchers did point out that the purities of some of the standard materials were not known with certainty.

Brennan et al.[80] report sensitivity in counts pg^{-1} for five arsenic compounds as follows: arsenite (40), DMA (24), MMA (17), arsenate (17), and *p*-arsanilic acid (19).

There is no comment made in the article as to why the values span this range. The researchers employed nano-HPLC-ICP-MS to separate standards solutions containing the compounds mentioned above.

1.6 Conclusions

After careful review of the literature, it is clear that gaps still exist in analytical methods for the determination of arsenic in rice. The IMEP study illustrates the difficulties in obtaining reliable data from validated methods. The data presented are highly variable, and in many cases the methods are not properly validated with respect to proper spiking procedures and evaluation of certified reference materials. Differing opinions with respect to drying samples prior to analysis can cause significantly erroneously reported results. Specific interferences and effects dealing with instrumental detection techniques for ICP-MS are also not often examined closely. With respect to arsenic speciation, HPLC separation methods are also in need of improvement. In many cases, inorganic arsenic species are indistinguishable, resolution between peaks is poor, and insufficiently encapped stationary phases contribute to peak tailing.

In light of these existing gaps, the purpose of the research presented in future chapters is to develop accurate and precise methods for the determination and speciation of arsenic in rice grain. In Chapter 2, for the determination of total arsenic in rice, special attention is paid to sample preparation drying procedures and ICP-MS effects such as acid concentration and carbon-loading. Chapter 3 describes improvements made to chromatographic HPLC separations, including the introduction of sample clean-up procedures. Chapter 4 addresses the general lack of information that is provided in the literature with respect to evaluation of current HPLC methods through calculations for resolution, peak tailing, and number of theoretical plates. Chapter 5 reports the conclusions and future research directions of the work proposed in this dissertation.

Table 1.1 Analytical methods for the determination of arsenic in various environmental and biological samples.

Sample	Method	Arsenic Species	Limit of Detection	Ref
Rice	Microwave Digestion HPLC-ICP-MS	total As	0.04	
Sea Water Wine Beer	GC-AED	inorganic As DMA MMA	0.8 (seawater, wine, beer) 25 (infant food) 0.05 (seawater, wine, beer) 1 (infant food) 0.15 (seawater, wine, beer) 10 (infant food)	[34]
Spiked Soils SRM 2711 Montana soil SRM 2709 San Joaquin soil	HPLC-ICP-MS	As(III) As(V) DMA MMA	0.1 0.15 0.12 0.13	[35]
Crude Fish oils	HPLC-ICP-MS	arsenolipids	0.5 ng cm^{-3}	[36]
Chards aubergines	HG-AFS	As(III) As(V) DMA MMA	3.1 3.0 1.5 1.9	[37]
Human Urine	HPLC-ICP-MS	As(III) As(V) DMA MMA ASB AsC	0.15 0.12 0.10 0.16 0.19 0.43	[38]
Arsine Gas	ICP-DRC-MS	arsine gas	0.10 µg m^{-3}	[39]
NIES Human Urine No.18 Cod Muscle (BCR 422) Dogfish Liver (DOLT 3) Dogfish Muscle (DORM 2) Fish Tissue (IAEA 407) Non-defatted Lobster Hepatopancreas (LUTS 1) Lobster Hepatopancreas (TORT 2)	Flow injection ICP-MS	total As	0.038 ng cm^{-3} with 0.3% nitric acid 0.062 ng cm^{-3} with 20 mM phosphate buffer	[40]

Table 1.2 Comparison of validation of methods for the speciation of arsenic in rice.

Spikes	Certified Reference Materials (CRM)	Drying Procedures	Inorganic Arsenic Species	Reference
N. I.	CRM_1		I_2	[73]
S_3	CRM_2		I_2	[71]
S_1	CRM_1, CRM_3	D_1	I_2	[72]
S_1	N. I.		I_1	[23]
N. I.	CRM_4		I_1	[32]
N. I.	CRM_1		I_2	[79]
N. I.	N. I.		I_1	[77]
S_3	N. I.		I_2	[67]
S_4	CRM_1		I_1	[49]

Spikes: S_1 (spike into rice sample matrix), S_2 (spike into extraction solvent), S_3 (aqueous standards only), S_4 (spike with only DMA or As(III) into rice sample matrix)

Certified reference materials: CRM_1 (SRM 1568a), CRM_2 (GBW 080001), CRM_3 (NIES no. 10), CRM_4 (GBW 10010) and CRM_5 (NMIJ 7503a).

Drying procedures: D_1 (samples dried prior to analysis)

Inorganic arsenic species: I_1 (inorganic arsenic species indistinguishable) and I_2 (inorganic arsenic species are separated)

N.I. (no information provided)

Table 1.3 Literature comparison of arsenic species determination in NIST SRM 1568a rice flour (certified reference value for total arsenic is 290 ± 30 ng g^{-1}).

Species sum	**Recovery (%)**	**As^{3+}**	**As^{5+}**	**DMA**	**MMA**	**Reference**
272.8 ± 9.9	94.1	63.4 ± 3.5	50.3 ± 2.9	144.2 ± 4.5	14.9 ± 3.9	[73]
286.4 ± 6.1	99.1 ± 2.1	129.2 ± 3.1	15.4 ± 3.8	31.5 ± 1.6	1.9 ± 0.7	[74]
281 ± 2	97.0	52 ± 1	44 ± 2	173 ± 2	12 ± 0.8	[43]
271 ± 3	93 ± 1	67 ± 5	36 ± 1	162 ± 1	5 ± 1	[7]
286.4 ± 6.2	82.3 ± 1.6	68.3 ± 3.7	20.5 ± 2.3	135.4 ± 4.1	8.1 ± 1.3	[70]
276	95.2	75	12	180	9	[53]
272	93.8	55 ± 6	41 ± 3	166 ± 6	10 ± 2	[67]
277	95.5	67 ± 4	39 ± 3	158 ± 5	13 ± 2	[75]
288.2	99.4	54.7 ± 1.4	53.7 ± 3.3	165 ± 8	14.8 ± 1.8	[76]
240 ± 40	80 ± 12	80 ± 14		160 ± 24	2	[77]
274	94	92 ± 4		174 ± 9	8 ± 2	[49]
290 ± 10	98.3	110 ± 10		180 ± 3		[78]
291 ± 19	100.1	119 ± 14	83 ± 6	78 ± 13	11 ± 6	**This study**

Concentrations shown in ng g^{-1}.

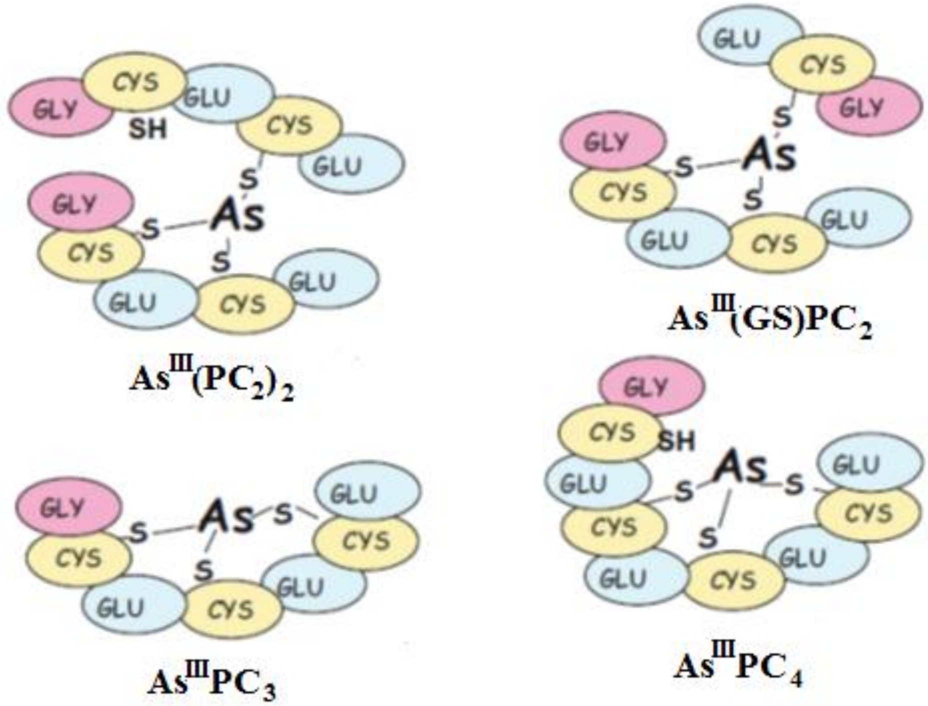


Figure 1 Structural representations of As PCs.[13]

CHAPTER 2

DETERMINATION OF TOTAL ARSENIC BY MICROWAVE-ASSISTED DIGESTION AND INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (ICP-MS)

2.1 Introduction

The optimization of a procedure that involves the analysis of a solid sample by an instrumental measurement following digestion to solubilize the analyte species is a nontrivial exercise, as there are numerous potentially interacting variables and several possible figures of merit to be considered. In principle, it should be possible to devise a digestion procedure in which all of the (nonarsenic) organic constituents of the sample are converted to volatile derivatives and all of the arsenic compounds are converted to a common chemical form that is dissolved. Following this, residual matrix (probably from the digestion reagents) could be removed by evaporation to dryness followed by dissolution in a matrix identical to that of the calibration standards. However, such a procedure, probably involving high-pressure, quartz-lined vessels and a suitable oven system, is likely to be both too expensive and too slow for routine laboratory use.

Microwave digestion offers a faster, more complete procedure for the dissolution of rice grains. As stated previously in Chapter 1, Section 1.3.3, closed vessels digestion systems are completed under high pressure and high temperature conditions that provide for more uniform, faster digestions with less contamination than tradition digestion block or hot plate methods.[48] Coupling these techniques with ICP-MS provides for a very sensitive technique with minimal instrumental interferences.

Methods for the determination of arsenic in environmental samples have been established in the literature, but many methods lack proper validation techniques. Many discrepancies also exist with sample preparation methods. Oven-drying biological materials is widely practice among the plant community.[41] Methods for drying rice grains involve temperatures from 80 ºC[42] up to 105 ºC[10]. The IMEP study[33] made a point to have laboratories analyze the samples on wet weight and to later correct for moisture content. The laboratories participating in the study found moisture contents varying from 0.5 – 14.4%. Nine laboratories did not correct there reported results for moisture content It is clear that several different methods are currently practiced. To ensure that no volatile species are lost during this process and to safeguard against the degradation of arsenic species in the sample, oven drying procedures must be evaluated.

In this chapter, an improved method for the determination of arsenic in rice grains is developed to address the need for a reliable method. The loss of arsenic due to sample drying procedures is reported, as well as significant effects with regards to acid concentration and carbon-loading with ICP-MS detection. The method was validated with appropriate spike recovery experiments and analysis of a certified reference material from NIST.

2.2 Research Objective

The goal of the research was to devise a procedure for the determination of total arsenic in rice grain that could be implemented with equipment likely to be widely available, such as the MARS Xpress microwave-assisted digestion system.

2.3 Experimental

2.3.1 Instrumentation

Samples were digested in 75 mL Teflon vessels in a CEM MARS Xpress microwave digestion system (Matthews, NC). Arsenic was determined with a Perkin Elmer Elan 6000 plasma source mass spectrometer (Shelton, CT). Operating conditions can be found in **Table 2.1**. Carbon studies were performed with a Perkin Elmer Optima 4300 DV plasma source optical emission spectrometer (Shelton, CT). Operating conditions can also be found in Table 1. Sample were ground in a Hamilton Beach Coffee Grinder (Southern Pines, NC) and ranges of particle size were selected by sieving through W.S. Tyler U.S.A. Standard Testing Sieves No. 18, 35, and 70 (Mentor, OH). 18 MΩ cm water was produced by a Barnstead E-pure system (Dubuque, IA). Rice digest solutions were filtered through 0.45 µm polyethersulfone membrane Whatman Puradisc syringe filters that were 25 mm in diameter. Volatile arsenic compounds released during drying were separated with a glass, gas-liquid separator (Perkin Elmer, Shelton, CT) and volatile compounds were trapped with liquid nitrogen in a coil 11 cm long, diameter of 1.5 cm with internal diameter of 2 mm.

2.3.2 Reagents

All solutions were prepared using 18.1 MΩ cm^{-1} water. Samples were digested with concentrated nitric acid (70% Trace Metal Grade) and hydrogen peroxide (30% certified ACS grade) from Fisher Scientific (Fair Lawn, NJ). A certified reference rice flour material, SRM 1568a, from NIST (Gaithersburg, MD) was used in the method validation. Standard arsenic solutions and spikes were prepared from sodium arsenate solid (sodium arsenate) from Fisher Scientific (Fair Lawn, NJ). Consumer rice was from Carolina Rice brand packaged by Riviana Foods Inc. (Houston, TX). Carbon standards were prepared from sucrose (certified ACS, saccharose) from Fisher Scientific (Fairlawn, NJ).

2.3.3 Samples

2.3.4 Analytical Procedure

Consumer white rice grain samples were blended as received and sieved below a particle size of 0.500 mm. An approximately 0.5 g sample was weighed into a 75 mL Teflon microwave digestion vessel, and 3 mL of concentrated nitric acid was added. The vessels were loosely capped and placed in the fume hood overnight. The following morning, the vessels were sealed, placed in the microwave oven, and heated 120 °C over 20 min by a linear temperature program. The vessels were held at 120 ºC for 10 min, cooled to room temperature, and slowly vented for over an hour. The resulting solutions were transferred to a 25 mL calibrated flask, diluted to volume. Prior to analysis the solutions were filtered through 0.45 µm syringe filters. Unless otherwise stated, samples were analyzed in triplicate, and an acid blank was analyzed for each data set.

Samples were analyzed as received. Moisture content was determined in a separate experiment, where 10 g samples were ground and sieved below 0.500 mm particle size, and dried in an oven overnight at 90 ºC. Subsequent arsenic concentration values reported were corrected for moisture content.

Solutions were analyzed for the arsenic content by ICP-MS at m/z 75. The instrument operating conditions are given in **Table 2.1**. External calibration was with matrix matched standards from a concentration range 0 to 10 µg g^{-1} arsenic as sodium arsenate that contained 9% nitric acid and 4000 mg L^{-1} carbon as sucrose. It was not necessary to match chlorine concentration in the samples, as the samples did not contain sufficient chlorine to cause a significant effect on the arsenic signal response.

The experimental design was based on the assumption that the response of the instrument was not affected by the digestion parameters. However, the amount of residual dissolved carbon may affect the extent of ionization of arsenic in the plasma and hence the sensitivity. Chlorine-containing reagents (such as hydrochloric acid) were not a good choice because of the $^{40}As^{35}Cl^{+}$ isobaric interference with arsenic at m/z 75. Effects related to acid concentration were also investigated.[55] For the initial experiments, the figure of merit was concentration (in the rice), as determined by calibration against non-matched standards, on the basis that the most relevant issues were incomplete dissolution and analyte loss, with a relative standard deviation of less than 20%. The digestion parameters investigated were particle size, sample mass, and various combinations of reagents, temperature programs, and venting procedures. The strategy adopted was a single cycle of the alternating variable procedure, on the basis that the parameters were largely independent of each other. Once a candidate method had been identified, the effects of residual matrix components on the signal were determined and a suitable calibration procedure was adopted. Finally, the method was validated by measuring recovery of arsenate spikes and the analysis of a certified reference material.

2.3.4.1 Sample Preparation

All rice grain samples, except the NIST SRM material, were homogenized in a coffee grinder and sieved to produce sample whose particles were in the size ranges 0 – 0.21, 0.21 – 0.50, 0.50 – 1.00 mm and greater than 1.00 mm. The effect of the particle size of the rice grains on the accuracy and precision of the results was tested. First, ten grams of whole rice grains were placed in the grinder, and ground with 3 short pulses. The ground sample was sieved through 1.00 mm mesh, and the rice remaining above the sieve was analyzed. All other smaller particle size portions of the sieved rice were discarded. The analyzed sample of particles above 1.00 mm were then reground and sieved through 0.50 mm mesh. The rice remaining above the sieve was analyzed, and so on until all particle sizes were analyzed. Three samples (0.500 g) for each size range were analyzed as described above. Sample masses of 0.10 g, 0.25 g and 0.50 g were digested in quadruplicate with particle size below 0.21 mm. Second, a new 10 g sample of whole rice grains was ground with 3 short pulses. All particle size portions added together. Three samples (0.500 g) were then analyzed. Lastly, a new 10 gram sample of whole rice grains was ground and sieved through 0.21 mm. Three samples (0.500 g) were taken from below the sieve, and subsequently analyzed. The standard deviations and 95% confidence intervals of the resulting ICP-MS intensity readings were calculated for each sample corresponding to its respective particle size.

Finely ground rice grains of differing sample masses were also evaluated for accuracy and precision with respect to arsenic concentration in the sample. A 10 gram sample of whole rice grains was ground and sieved through 0.21 mm. Sample masses of 0.100 g, 0.250 g and 0.500 g were analyzed in triplicate. The standard deviations and

95% confidence intervals of the resulting ICP-MS intensity readings were calculated for each sample corresponding to its respective sample mass.

Samples were analyzed as either “dry”, meaning that the samples were dried overnight in an oven at 90 °C, or “wet”, meaning that the samples were analyzed as received. The particle size was below 0.21 mm and the sample mass was 0.5 g. Any loss of arsenic was calculated from differences between the concentrations found for the “wet” samples and those found for the “dry” Carolina Rice samples.

The volatile arsenic species were trapped in a liquid nitrogen cold trap. Approximately 10 g of rice was heated in a hot water bath at 100 °C for 7 h. Air was allowed to flow from the headspace to a plastic coil by a peristaltic pump. The resulting vapors were collected in the coil via a liquid nitrogen cold trap. The arsenic was concentrated in the cold trap, and then released by removing the coil from the liquid nitrogen bath and allowing the coil to warm to room temperature in air. The coil was connected to the ICP-OES instrument, and the sample was directed into the instrument by a peristaltic pump. Calibration of the instrument was performed with arsine gas standards, generated from hydride generation from arsenite solution standards and 0.2 % sodium borohydride in 0.05 % sodium hydroxide solution. The solutions were pumped together into a gas-liquid separator by a peristaltic pump. The liquid was discarded to waste, while the gas was allowed to accumulate in a plastic coil. Similar signal response curves were generated for gas standards and trapped volatiles from the Carolina Rice sample.

2.3.4.2 Microwave-assisted Digestion

The effects of acid concentration, the use of peroxide, temperature programs, time, and venting were investigated. For each parameter, 0.5 g Carolina Rice sample were placed into a 75 mL Teflon CEM microwave vessel. The volume of concentrated nitric acid added to each vessel was either 3 mL, 4 mL or 5 mL of nitric acid. Hydrogen peroxide effects were also studied by repeating the analysis with addition of 2 mL to each reaction vessel. Spikes (sodium arsenate), 0.5 mL of 250 µg L^{-1} were added to the vessels prior to the digestion procedures for all of the analyses. The effect of temperature was investigated for 0.5 g blended and sieved Carolina Rice sample and 3 mL of concentrated nitric acid. Vessels were heated to 120 °C, 140 °C or 160 °C, over a 20 min period, then held at the maximum temperature for 10 min, and cooled for 1 hour. The vessels were vented by slowly unscrewing the caps of each vessel. The solutions were vented immediately after cool down, one hour after cool down, and after standing overnight.

2.3.4.3 Detection of Arsenic by ICP-MS

The effect of nitric acid concentration on arsenic signal response in ICP-MS was studied. Standard 10 µg L^{-1} arsenic solutions were prepared with nitric acid concentrations varying from 0 % to 12 % (v/v) in 1 % increments. The percent change in the arsenic signal response intensity was plotted as a function of percent acid concentration versus with respect to a zero percent acid standard. The acid concentration after digestion was determined by titration with standard 1.0 M sodium hydroxide solution.

The post-digest carbon concentration was determined against sucrose standards in 9% nitric acid by ICP-OES at carbon wavelength 188.979 nm. Standard solutions were prepared with 10 µg L^{-1} arsenic in varying concentrations of carbon added as sucrose from 0 to 4200 mg L^{-1}. Carbon concentration was plotted versus percent change of arsenic signal response intensity with respect to a zero carbon standard.

According to the certificate for SRM 1568a, the non-certified mass fraction of chlorine (as chloride) in the rice flour is 300 mg kg^{-1}. Tsukada and Takada[50] determined the chlorine concentration of polished white rice to be 140 mg kg^{-1}. The effect of chlorine (added as sodium chloride) concentrations in increments of 5 mg L^{-1} up to 20 mg L^{-1}, on the response to a 10 µg L^{-1} arsenic standard solutions, was measured.

2.3.4.4 Validation

Predigestion spikes, consisting of 0.5 mL of 250 µg L^{-1} As (as arsenate), were added to the microwave reaction vessels together with the rice grain sample and digestion reagents. This is equivalent to the addition of 5 µg L^{-1} As to the diluted, post-digest solution. Percent recoveries were calculated for each digestion procedure.

NIST certified reference material SRM1568a, rice flour containing 290 µg kg^{-1} arsenic was analyzed by calibration against matrix matched standards and also by standard additions. The slopes of the calibration lines were compared.

2.4 Results and Discussion

2.4.1 Sample preparation

The effects of particle size are shown in **Table 2.2** from which it can been seen that the best approach for good precision is to grind whole rice grains down to a particle size below 0.21 mm. It can be seen that as the particle size of the rice gets smaller, the reported arsenic concentration values increase. This could be due to better efficiency of the microwave digestion on smaller particle sizes. When all particle size portions are analyzed together, a significantly lower arsenic concentration (138 ng g^{-1}) is found. Therefore, it is recommended that rice grain samples should be ground, all together, down to a particle size below 0.21 mm. This procedure resulted in the largest reported value of arsenic (345 ng g^{-1}) in the rice samples.

The effects of sample mass on precision are shown in **Table 2.3**. The particle size for these samples was below 0.21 mm. Due to sample dilution for ICP-MS analysis, 0.100 g sample size resulted in arsenic values lower than the detection capabilities of the instrument. The standard deviation for a sample size of 0.250 g was 50 % of the total arsenic content in the rice. For 0.500 g samples, the standard deviation between samples was only 18 % of the total arsenic content. A sample size of 0.5 g was considered sufficient to be an accurate representation of the bulk material if the particle size was less than 0.5 mm.

The results for the effects of drying are shown in **Table 2.4**. Oven drying at 90 ºC resulted in an 11 % loss of arsenic relative to samples that were not dried. This would appear to be an important finding that has not, to our knowledge, been reported before.

Although the exact arsenic species lost during this step was not identified, it can be postulated that this species is a methylated arsine compound arising from the reduction of DMA. According to the Challenger mechanism,[81] DMA can be reduced to dimethylarsine, then methylation/oxidation produces trimethylarsine oxide (TMAO) which can further be reduced to trimethylarsine (TMA), both of which are volatilizable compounds. The reaction mechanism can be found in **Figure 2.1**. The challenger mechanism requires a reducing agent and methylating agent. According to Cullen et al.,[81] glutathione can act as a reducing agent in the formation of this volatile compound and s-adenosylmethionine acts as the methylating compound. Rice itself could possess these compounds naturally, or more likely residual chemicals from bacteria are left in the rice matrix that are capable of aiding the formation of volatile arsenic compounds. Rosen et al.[83] report that when s-adenosylmethionine, glutathione and the ArsM enzyme (found in bacteria) are in the presence of either DMA or As(III), both TMAO and TMA are formed. In fact, a decrease in the As(III) peak of the HPLC chromatogram correlated with the production of DMA, TMAO and TMA. This enzyme was found to accelerate the methylation process and could also be present in the rice matrix from bacterial residues.

The boiling point of TMA is 52 ºC, while the boiling point of DMA exceeds 200 ºC.[82] While it is unlikely that DMA would degrade at oven drying temperatures at 90ºC, the transformation of DMA to TMA does result in a significantly lower boiling point. The boiling point of TMAO is 170 ºC. At higher drying temperatures, TMAO is volatilizable. Methods for the identification of this volatile compound are discussed in Chapter 5.

2.4.2 Microwave-assisted Digestion

The results for the various oven programs are shown in **Table 2.5**. The Carolina rice samples (0.5 g and <0.21 mm particle size) were analyzed. The greatest concentration was obtained at 120 °C. It was also observed that low values were obtained if the vessels were vented before being cooled to room temperature. Together with the results concerning the loss of arsenic on drying, it is concluded that some fraction of the arsenic in this rice sample is volatile and is lost if suitable precautions are not taken.

The results for various digestion procedures are shown in **Table 2.6**. Digestions with hydrogen peroxide and nitric acid produced solutions that had lower arsenic concentrations (by about 33%) than those from the same material digested with nitric acid alone. The solutions obtained by digestion without the overnight steeping had the same concentration of arsenic as those that had been digested overnight, but had higher concentrations of residual carbon, which caused interferences in the ICP-MS measurement. Overnight digestions without venting prior to the microwave program (i.e. microwave vessels were sealed throughout the entire digestion process) produced low arsenic concentrations, whereas overnight digestion with venting prior to the microwave-assisted digestion gave the expected concentrations. This is difficult to interpret, given that the presence of a volatile arsenic compound is indicated from the results of other experiments. It may be related to the vapor pressure of the volatile compound and the total pressure in the vessel.

2.4.3 Detection of Arsenic by ICP-MS

The effect of nitric acid concentration on the signal for a 10 µg L^{-1} standard solution is shown in **Figure 2.1** from which it can be seen that the arsenic signal response varies between 92% to 109%,. Acid concentrations from 3% (v/v) to 8% (v/v) produced signal responses with less than 2% difference. Below 3% (v/v) and above 8% (v/v), acid concentration has a significant effect on the arsenic signal response, up to 9% difference. The acid concentration in the digests, determined by acid-base titrimetry was 9% (v/v) and so the standards were matched to the samples in terms of concentration by the addition of sufficient acid so that the final concentration was 9% (v/v) nitric acid.

The effect of carbon concentration on arsenic signal response for a 10 µg L^{-1} standard solution is shown in **Fig. 2.2**. As the carbon (added as sucrose) content increased from 0 to 4200 mg L^{-1}, the relative arsenic signal response increased from 100% to 165%. After acid digestion, rice starch will become glucose and glucose-based polymers.[66] For the purpose of this study, sucrose was used as the carbon source in standard solutions. The concentration of carbon in the diluted digest sample solution was determined (by ICP-OES) to be 4000 mg L^{-1}, and so the calibration standards were matched to the samples by the addition of sufficient sucrose to produce a carbon concentration of 4000 mg L^{-1}.

As discussed previously in Chapter 1, carbon is known to enhance the ionization intensity of elements in the plasma through a proposed charge transfer mechanism from C^{+}-species to the analyte atom, resulting in an increased signal response for the analyte. Researchers have exploited this finding by introducing carbon-containing compounds such as methanol into the plasma in order to increase the sensitivity of the arsenic signal response and therefore lower the limit of detection for the method. Adding a carbon source does, however, create unwanted build-up of carbon on the instrument cones and lens which can effectively decrease instrument sensitivity over time. It is therefore not recommended that high concentrations of carbon be added into the plasma. In any case, it is necessary to matrix match the standard solutions to the sample with respect to carbon concentration so that any signal enhancement is accurately compensated. If standard calibration solutions are not matrix-matched, artificially high concentrations of arsenic in the sample material will be reported.

The effect of chlorine on the arsenic response is shown in **Figure 2.3**. As can be seen, the change is relatively small, the signal increased from 100% to 108%. As it is expected that the chloride concentrations in the digest will be only a few mg L^{-1}, the standards were not matched with respect to the chlorine content of the samples.

2.4.4 Validation

The concentration of arsenic in SRM 1568a (rice flour) was determined to be 301 ± 59 mg kg^{-1}, in agreement with the certified value of 290 ± 30 mg kg^{-1}. The comparison of the slopes of the matrix matched calibration and the standard additions calibration were compared. The slope of the standard addition calibration (4200 counts per second per µg L^{-1}) for the SRM material is not significantly different from that of the external calibration (4282 counts per second per µg L^{-1}).

The spike recoveries for 100 ng of arsenic added as sodium arsenate are given in **Table 2.6**. It may be seen that the results are somewhat inconsistent, though omission of a venting stage does apparently lead to low recovery.

2.5 Conclusions

Although suggestions for tolerable intake of arsenic in foodstuffs have been proposed, no regulations have been established thus far. As the literature reports continually increasing publications surrounding the determination of arsenic in rice, it becomes inevitable that such regulations will be needed in the near future. The first step in establishing these laws is to ensure that the analytical methods for the determination of arsenic in foodstuffs are reliable and accurate.

Recently, the difficulty in providing sound analytical methodology was highlighted in the results from a proficiency test set up for the determination of total arsenic and inorganic arsenic in rice. In the IMEP-107 study[33] researchers attempted to homogenize a rice sample as part of a study in which the bulk rice material was homogenized and distributed to the participating laboratories. According to the original lab, the samples were homogeneous for both total and inorganic arsenic and stable for over 6 weeks. Reference values for total arsenic content established by 7 expert laboratories were reported in $\mu g\ kg^{-1}$ as 164, 190, 176, 139, 172, 190 and 171. The range of these values is substantial, considering that the labs undertaking the project were considered experts in analytical methodology related to the determination of arsenic in rice. After this analysis, the sample was sent out to 103 separate laboratories from 35 different countries. Of the labs that participated in the study, 59% performed satisfactorily, 16% of the labs reported questionable results, and 25% of the laboratories performed unsatisfactorily. It can be seen from the report that there was a wide range of results with a high percentage of the laboratories scoring outside of the acceptable range. It should be recognized that this sample was homogenized and stable. This study highlights the difficulty of accurately measuring arsenic content in rice, and the need for more robust methods for the determination of arsenic in rice.

The method proposed in this Chapter, for total arsenic determination in rice by microwave digestion followed by ICP-MS, is an accurate method validated with solution spikes into the rice matrix, analysis of SRM 1568a (rice flour), and similar sensitivity values between the matrix-matched standards and the standard additions curve. Many current methods do not properly validate their procedures, and often times no mention of spike recoveries or matrix-matching standard solutions prior to ICP-MS detection. Garnier et al.[84] dry their samples overnight in an oven at 70 ºC, and validate their methods by inserting reagent blanks into their run, and through the analysis of NIST SRM 1568a. No mention is made of any spike recovery experiments. The results of the analysis for SRM 1568a were also not reported. Fontcuberta et al.[85] microwave digest their samples up to 200 ºC employing nitric acid and hydrogen peroxide. To validate their method, they correctly spike standard solutions into the rice matrix. The analysis of SRM 1568a was 275 ± 15 µg kg^{-1} (certified value 290 ± 30 µg kg^{-1}). The digestion parameters investigated in this dissertation were particle size, sample mass, and various combinations of reagents, temperature programs, and venting procedures. Evidence from experiments point out that high temperature, closed vessel microwave digestion procedures involving hydrogen peroxide lead to low recovery of arsenic in the final solution. Fontcuberta et al.'s[85] method involves both hydrogen peroxide and very high digestion temperatures. This may explain why recovery of SRM 1568a was a little low.

In order to avoid artificially low results, special attention must be paid to the drying procedures in the analytical methods. Previous methods have not been vigilant with respect to ensuring that no arsenic is lost during the drying procedure. Drying plant materials in an oven prior to analysis is a common practice reported in the literature. As previously stated in section 1.3.2, drying temperatures can vary from 80 ºC[42] to 105 ºC.[10] From the results obtained in this work, any methods that contain a drying step should be recognized as questionable. It is also important to note that spike recovery experiments usually begin directly prior to digestion procedures, neglecting to evaluate arsenic loss due to oven drying sample preparation.

To avoid reporting artificially high results, careful attention must also be paid to matrix-matching the standard calibration solutions with respect to acid concentration and carbon concentration. This information is often not readily available in the literature. There is one study that referenced carbon-loading effects in the plasma, and corrected for those effects. Dufailly et al.,[86] buffered the carbon effect on the arsenic signal by adding isopropanol to all of their solutions. This is the only paper that makes reference to adjusting for carbon concentrations. In order to reduce possible contamination issues arising from the addition of a solution into your sample, a more effective method is matrix-matching standard solutions with respect to carbon concentrations.

The methods proposed in this chapter are correctly validated with spikes into the rice matrix and analysis of SRM 1568a. Samples were analyzed on wet weight, to avoid the loss of volatilizable arsenic compounds lost in the oven-drying step. Matrix matching of standard solutions, with respect to acid concentration and carbon concentration, is a necessary component when ICP-MS is the detection technique.

Table 2.1 Operating conditions for ICP-MS and ICP-OES

ICP-MS	
Sample Introduction	GemTip Crossflow II nebulizer
Spray Chamber	Scott spray chamber
Plasma Gas Flow	15 L/min
Auxiliary Gas Flow	0.2 L/min
Nebulizer Gas Flow	0.8 L/min
RF Power	1300 W
Pump Flow Rate	1.5 mL/min
As 74.9216 amu	
ICP-OES	
Sample Introduction	GemCone nebulizer
Spray Chamber	Glass cyclonic spray chamber
Lens Voltage	8 V
Pulse Stage Voltage	1600 V
Nebulizer Gas Flow	1.03 mL/min
RF Power	1500 W
C 193.030 nm	

Table 2.2 Total arsenic determination at varying particle sizes for 0.500 g Carolina Rice sample.

Particle Size (mm)	**Mean arsenic concentration (ng g^{-1})**	**Standard deviation (ng g^{-1})**	**95% Confidence Interval**
x > 1.00[a]	139	15	25
0.500 < x < 1.00[a]	108	2	3
0.21 < x < 0.500[a]	179	20	34
x < 0.21[a]	263	23	39
Quick grind/all fractions[b]	138	5	8
x<0.21[c]	345	39	66

[a] Whole grains ground to sequentially smaller particle sizes. Particle lying below each designated sieve were discarded, while particles remaining above sieve were analyzed. [b] Whole grains were quickly ground, all particle size portions were analyzed together. [c] Whole grains were ground down to a particle size less than 0.21 mm.

Table 2.3 Total arsenic determination with varying sample masses of Carolina Rice sample analyzed in quadruplicate.

Weight (g)	Mean arsenic concentration (ng g^{-1})	Standard deviation (ng g^{-1})	95% Confidence Interval
0.100	n.d.	n.d.	n.d.
0.250	140	69	110
0.500	218	38	60

*n.d. means data not determined because the values were below the detection capabilities of the instrumental method.

Table 2.4 Determination of total arsenic in dry (samples dried in an oven to constant weight) and wet (samples analyzed as received). Values include correction for moisture content in rice.

Sample	Arsenic concentration (ng g^{-1})	Mean arsenic concentration (ng g^{-1})
Wet 1	435	415
Wet 2	366	
Wet 3	443	
Dry 1	364	371
Dry 2	371	
Dry 3	378	

Table 2.5. Determination of total arsenic in Carolina rice sample at varying microwave assisted digestion temperature programs, each with a 20 min ramp time and 10 min hold time.

Temperature Maximum (°C)	**As concentration in rice (ng g^{-1}) at 95% C.I.**
120	217 ± 64
140	124 ± 33
160	109 ± 50

Table 2.6. Microwave digestion conditions with a 20 min temperature ramp to 120 °C and hold at 120 °C for 10 min. The table shows same day versus overnight venting procedures.

	3 mL HNO_3			
	Same day	Overnight with vent	Overnight without vent	Overnight no T° program
Avg. conc. (ng g^{-1})	290	303	308	320
Spike Recovery (% v/v)	104	108	54	24
	3 mL HNO_3 and 2 mL H_2O_2			
	Same day	Overnight with vent	Overnight without vent	Overnight no T° program
Avg. conc. (ng g^{-1})	229	199	173	261
Spike Recovery (% v/v)	104	79	101	75

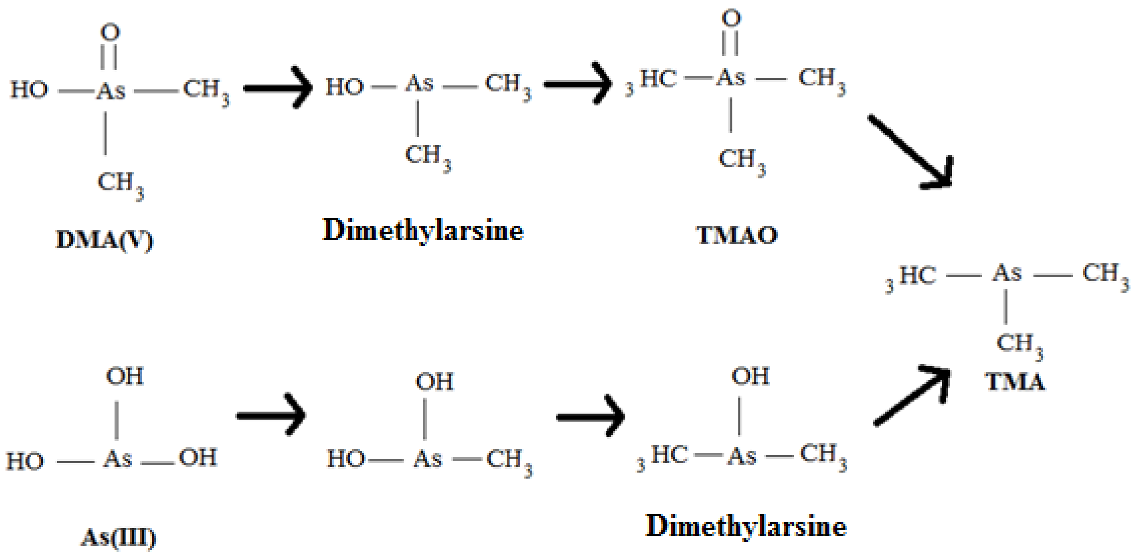


Figure 2.1 The reaction mechanism of arsenic volatilization as illustrated by Challenger and Cullen.[82]

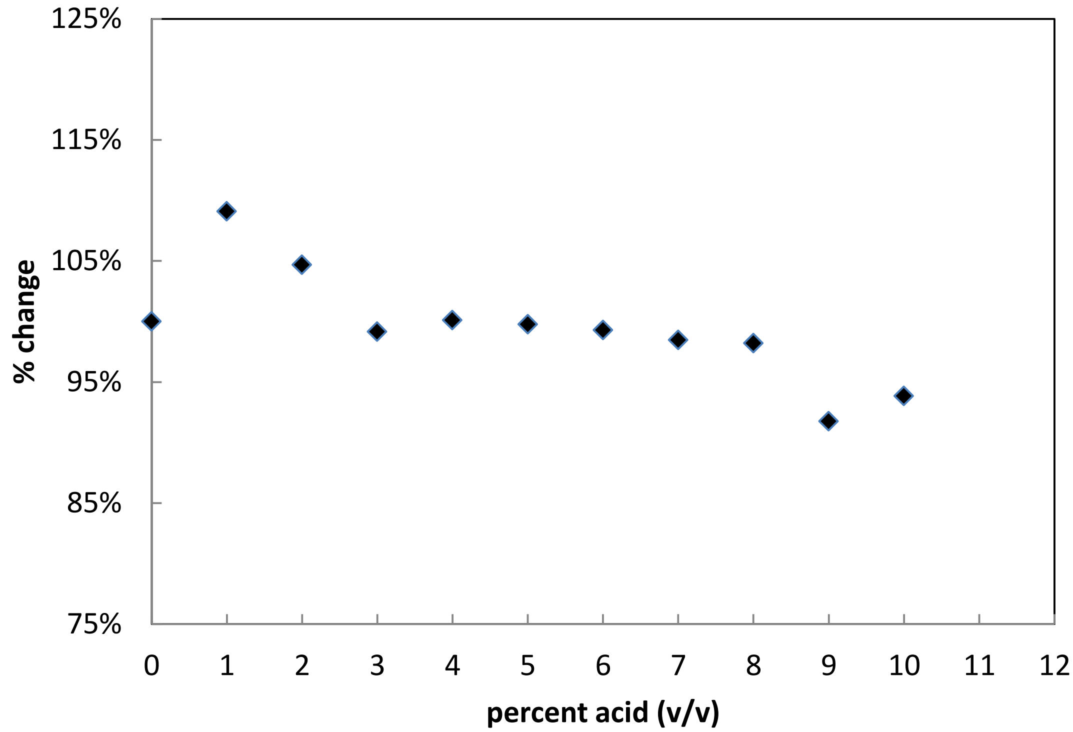


Fig. 2.2 Effect of acid concentration on 10 µg L^{-1} arsenic signal response in ICP-MS.

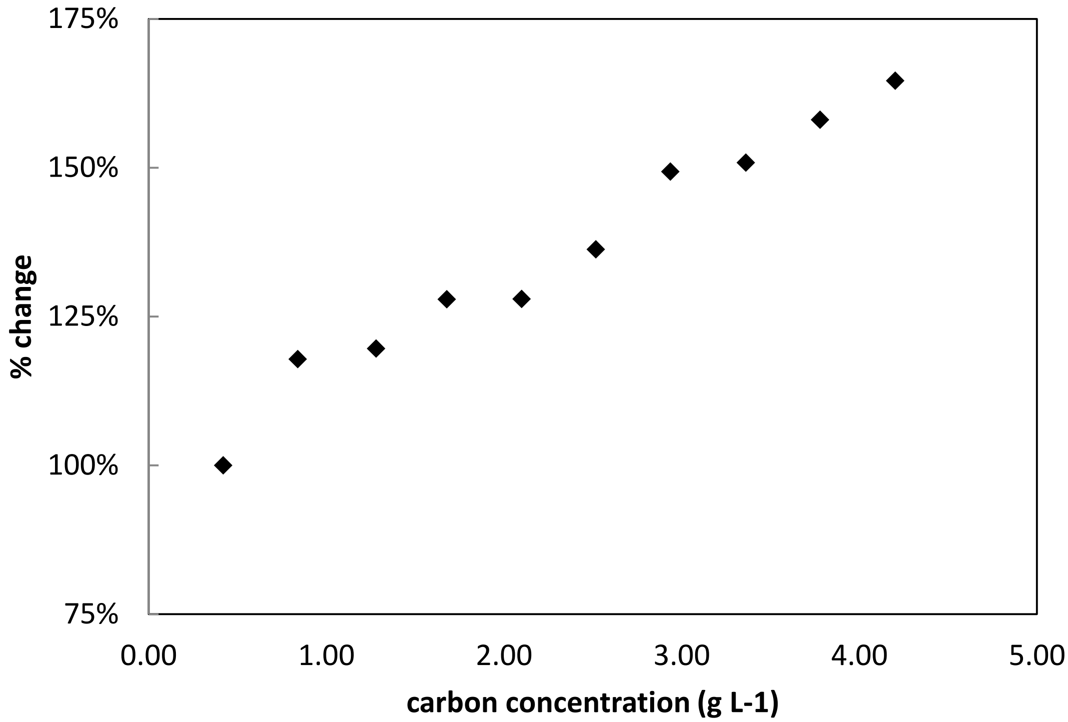


Fig. 2.3 Carbon loading effects on 10 µg L^{-1} arsenic signal response in ICP-MS.

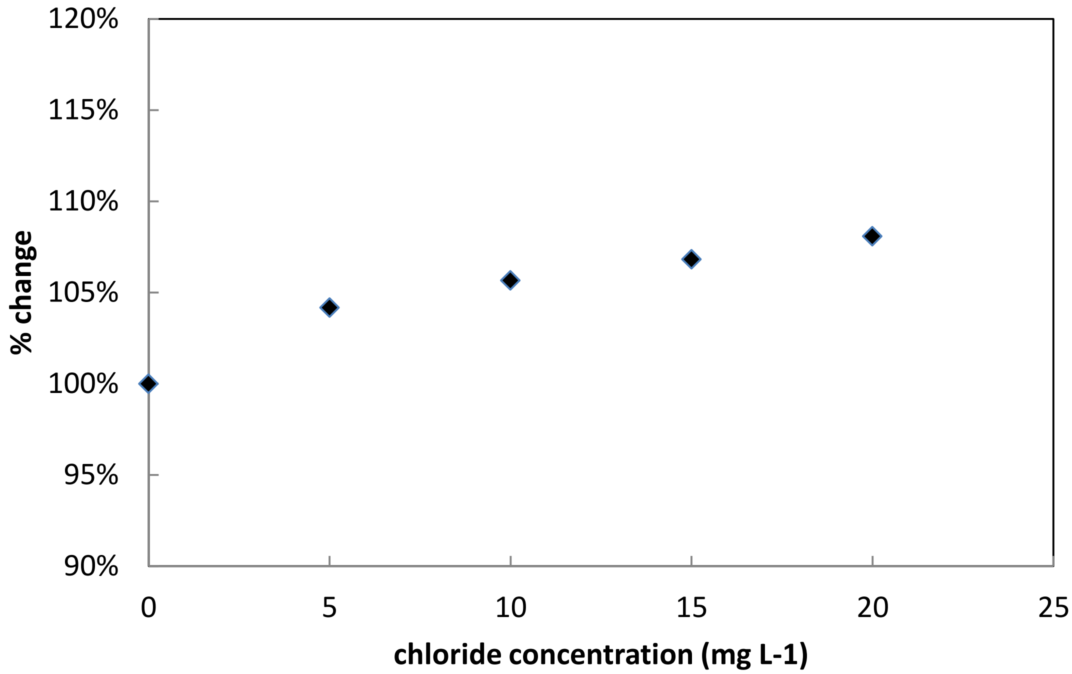


Fig. 2.4 Effect of chloride on 10 µg L^{-1} arsenic signal response in ICP-MS.

CHAPTER 3

DETERMINATION OF ARSENIC SPECIES IN RICE BY HPLC-ICP-MS

3.1 Introduction

The International Union for Pure and Applied Chemistry (IUPAC)[87] has defined the following words related to speciation analysis:

> i. *Chemical species*. Chemical elements: specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure
> ii. *Speciation analysis*. Analytical chemistry: analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample
> iii. *Speciation of an element; speciation*. Distribution of an element amongst defined chemical species in a system

In a recent review,[64] it was stated, "establishing the location and speciation of arsenic within the edible rice grain is essential for understanding the risk and for developing effective strategies to reduce grain arsenic concentrations."

Arsenic speciation in rice grain material is not a trivial exercise. There is no universal HPLC separation that is widely used. Extraction conditions vary with respect to extract solutions, heating time, and the use of physical methods, such as sonication, to aid the process. Many HPLC procedures do not take the high starch content of rice into account in their analyses, therefore, possibly damaging the chromatographic columns, along with decreasing peak resolution. The originality of the methods is often not discussed, with many authors quoting previous works rather than developing novel improvement.

HPLC separations of arsenic compounds began in the early 1990s with anion-exchange columns such as the Hamilton PRP-X100[88] or with other weak-anion exchange resins such as the Adsorbosphere-NH_2.[89] A typical chromatogram can be seen in **Figure 3.1** (a). Bavazzano, et al.[89] separated 5 arsenic species in human urine samples with a 5 µm column packed with Adsorbosphere-NH_2 weak anion exchange resin. A mobile phase consisting of 10% methanol in 15 mM $NH_4H_2PO_4$-1.5 mM CH_3COONH_4, pH adjusted with CH_3COOH to 5.75 was used. Over the course of 30 years, little advancement was made to arsenic speciation analysis. Castillo et al.[90] recently employed a PRP-X100 column for the separation of the same 5 arsenic compounds in human urine (**Figure 3.1** (b)). Although the elution time has improved slightly, peak tailing and poor resolution between peaks can still be seen. Many of the same columns like the PRP-X100 are commonly used in separations today and have clearly not advanced the state of arsenic speciation analysis.

In order to improve upon HPLC separations for arsenic speciation, alternative stationary phases with improved surface functionalities should be investigated. Previous works employ the anion-exchange column PRP-X100. This column has been used since the inception of HPLC arsenic speciation analysis, and has not been improved with respect to their surface chemistry related to the effective separation of arsenic species. Reversed phase liquid chromatography (RPLC) involves the separation of compounds by a non-polar stationary phase and a polar mobile phase. The separation of analyte compounds occurs with analyte partitioning between the stationary and mobile phases. Separations are also influenced by the packing particle’s pore size, and how efficiently the molecules can move in and out of them. As illustrated in **Figure 3.2**, these stationary phases contain silica-based particles with open pores. The surfaces of these particles are modified with specific functional groups, in this case a C8 chain. This carbon chain acts as an organic “skin” attached to the stationary phase. Ideally, the entire surface of the particles should be covered with alkane groups. Free silanol groups that have not reacted with the C8 bonded phase accumulate throughout the surface as well. These silanol groups can cause peak tailing. To improve peak shape, these silanol groups should be capped with methyl groups or other small functional groups.

In addition to changes to the stationary phase surface, ion-pairing reagents can also be employed to improve upon the separation. In ion-pairing, the solute ion is paired with a counter ion possessing an extended non-polar group chain. Once the two are paired, the hydrocarbon chain interacts with the stationary phase. These secondary interactions can improve resolution between chromatographic peaks. In this work, the use of tetrabutylammonium hydroxide ion-pairing reagent significantly improves arsenic spearations on the Sunfire C8 column. The anionic arsenic species (MMA and As(V)) in pH 5.80 are successfully paired with this compound. The remaining butyl groups interact with the stationary phase to effectively separate the DMA and MMA peaks with better resolution than previously published work.

This chapter describes the separation of As(III), DMA, MMA, and As(V) on a C8 column that has improved surface functionality of the stationary phase. Residual silanol groups have been masked, to create better separations with respect to peak shape and resolution. To further improve the separations, an ion-pairing reagent, tetrabuytlammonium hydroxide (TBAH) in 1.3% methanol adjusted to pH 5.80 with malonic acid was added to the mobile phase. The structure of these compounds can be found in **Figure 3.3**. The chemical forms of As(III), DMA, MMA, and As(V) can be found in **Figure 3.3**. One would expect As(III), which is fully protonated and uncharged, to elute from the column first. DMA is also an uncharged species at pH 5.80, however, the methyl group located within the molecule can slightly interact with the C8 stationary phase. MMA and As(V) are charged species at pH 5.80, and will therefore pair with TBAH as they move through the column. DMA contains methyl groups which may cause a stronger interaction between itself and the carbon chains on TBAH. This may cause some steric hindrance of the TBAH molecule while interacting with the stationary phase. DMA would therefore be the third compound to elute from the column. As(V) is charged at pH 5.80, and will pair with TBAH and elute last from the column.

A dialysis clean-up step was also investigated to help column efficiency and column life. Rice contains large, bulky starch material consisting of amylose and amylopectin polymer chains. It is possible to degrade these starch molecules with enzymes such as α-amylase or gluco-amylase. These enzymes hydrolyze the starch molecules, breaking them down into smaller glucose molecules. Although the starch molecules are sufficiently hydrolyzed enough to pass through a chromatographic column, this option still allows for significantly high amounts of glucose to be injected onto the column. Filtering sample digests before they are injected onto the column provides for a cleaner sample. However, evidence presented in this chapter suggests that a substantial amount of arsenic can be lost to the filter. Dialysis allows for arsenic species to equilibrate through the pores over time. This results in a cleaner sample without arsenic loss.

3.2 Research Objective

The objective of this research was to develop a validated, improved method for the speciation of As(III), As(V), DMA and MMA in rice grain. Such a method would involve an extraction procedure does not alter the chemical species involved and a sample clean-up procedure that protects both the chromatographic performance and column life. The method was validated by spiking standard solutions into the rice matrix, and the analysis of SRM1568a, together with a comparison with values for the speciation of this material already reported in the literature.

3.3 Experimental

3.3.1 Instrumentation

Arsenic was extracted from the samples in 75 mL Teflon vessels in a CEM MARS Xpress microwave digestion system (Matthews, NC). Arsenic was determined with a Perkin Elmer Elan 6000 plasma source mass spectrometer (Shelton, CT). Operating conditions can be found in **Table 3.1**. Chromatography was performed on a Sunfire C8 column, 5 µm, 4.6 x 20 mm with a Sunfire guard column (Waters Corporation, Milford, MA). Chromatographic conditions can be found in **Table 3.1**. Chromera chromatographic software (Perkin Elmer, Shelton, CT) produced information related to chromatograms. Samples were ground to a particle size less than 0.212 mm in a Hamilton Beach Coffee Grinder (Southern Pines, NC), and sieved through W.S. Tyler U.S.A. Standard Testing Sieve (Mentor, OH). 18 MΩ cm water was produced by a

Barnstead E-pure system (Dubuque, IA). Rice digest solutions were dialyzed through cellulose membrane dialysis tubing, average flat width 25 mm from Sigma-Aldrich (St. Louis, MO).

3.3.2 Reagents

All solutions were prepared using 18.1 MΩ cm^{-1} water. Standard arsenic solutions and spikes were prepared from sodium arsenate certified and sodium arsenite (meta) from Fisher Scientific (Fair Lawn, NJ), disodium methyl arsonate hexahydrate, purity 97.5% HPLC grade from Chem Services (West Chester, PA), and cacodylic acid, 98% from Sigma (St. Louis, MO). Mobile phase for HPLC separations was prepared from tetrabutylammonium hydroxide, 1.0 ¡M solution in methanol from Sigma-Aldrich (St. Louis, MO). The pH of the mobile phase was adjusted with malonic acid, Reagent Plus 99% from Sigma-Aldrich (St. Louis, MO).

3.3.3 Samples

A certified reference rice flour material, SRM 1568a, from NIST (Gaithersburg, MD) was used in the method validation. Consumer rice was from Carolina Rice brand packaged by Riviana Foods Inc. (Houston, TX).

3.3.4 Analytical Procedure

For each experiment, 10 g Carolina rice was homogenized by grinding and sieving rice grains to a particle size less than 0.212 mm. 0.5 g portions were weighed into the microwave vessels and 5 mL distilled, deionized water was added. The vessels were capped and sealed, then placed in the microwave in a temperature ramping program to 95 ºC for 5 min and held for 90 min. The resulting gelatin was diluted, while still hot, to 15 mL with d_i water. (The solution is easier to transfer when still hot, because the starch materials in the rice extract stay in solution at higher temperatures. If the extracts are cooled to room temperature, a gel will form.) The extracts were then transferred to the dialysis tubing, and the ends of the tubing were tied off. The filled bag was placed into an Erlenmeyer flask containing 200 mL water. The solution was boiled for 2 h. The dialysis bag was then removed from the flask. The resulting solution was boiled down to about 25 mL. This final solution was injected directly onto the HPLC column. The method was validated with solution spike studies into the sample matrix and mass balance. Literature values for arsenic species present in SRM 1568a were also compared.

3.3.4.1 Extraction of Arsenic

The efficiency and reliability of nitric acid[66] and water to extract arsenic species from solid rice grains were investigated. Comparisons between clean-up procedures (dialysis and centrifuging) were also accomplished. The centrifuging procedure involved centrifuging the sample extracts at 5000 rpm for 10 min, after the microwave-assisted extraction. The supernatant was collected for analysis, while the solid gel material left at the bottom of the tube was washed with 10 mL water. The solution was mixed by shaking the tube vigorously, and the resulting mixture was then centrifuged for a second time. These centrifuging and washing steps were completed 3 times. Subsequently, all three supernatants were added together into a final combined solution which was injected onto the chromatographic column.

3.3.4.2 Sample Clean-up Procedures

Dialysis procedures were optimized with respect to dialysis time. A 15 mL standard solution of 24 µg L^{-1} each of As(III), DMA, MMA, and As(V) was added to a dialysis bag. The bag was tied off, and added to an Erlenmeyer flask containing 200 mL of d_i water. The solution was boiled for 3 h. Every 30 min, 1 mL of solution outside of the bag was taken for subsequent injection onto the analytical column. The volume of the boiling solution was maintained throughout the experiment.

To examine the effect of the matrix on dialysis time, a rice extract sample was carries through the dialysis procedure. The bag was boiled for 3 hours total. The liquid remaining outside of the bag was then discarded. The Erlenmeyer flask was re-filled with 200 mL water, and left to sit overnight to ensure that most of the arsenic in the rice extract had been dialyzed out of the bag. The following morning, the bag was removed from the solution and washed with water. The bag was then carefully opened. A 0.5 mL volume of a standard solution of As(III), DMA, MMA and As(V) was added to the inside of the bag so that the final concentration inside the bag was 24 µg L^{-1} for each standard. The bag was subsequently tied off and added to an Erlenmeyer flask containing 200 mL of d_i water. The solution was boiled for 3 hours. Every 30 minutes, 1 mL of solution outside of the bag was taken for subsequent injection onto the analytical column. The volume of the boiling solution was maintained throughout the experiment by adding water back to the boiling solutions periodically.

Clean extract solutions were also evaluated for carbon content by ICP-OES to determine whether the dialysis procedure decreased the amount of carbon compounds in the solution. The final extract solutions were run on the ICP-OES against standard sucrose solutions.

3.3.4.3 Separation and Detection by HPLC-ICP-MS

HPLC parameters were optimized with respect to mobile phase pH, flow rate, and injection volume. Mobile phase pH was varied from 2 to 10 to determine the optimal pH for the separation. The resulting chromatograms were evaluated with respect to peak resolution, total elution time, and elevated background signal to noise. Optimization of the flow rate was completed by comparing the chromatographic merits listed above. The flow rate was varied from 0.8 mL min^{-1} up to 1.4 mL min^{-1}. Several injection loop volumes were also investigated, including 20 µL, 50 µL and 100 µL sizes.

For each injected solution, the m/z 35 (chlorine) was monitored simultaneously with the m/z 75 (arsenic) to ensure that the elution time of the chloride peak did not overlap was any arsenic-containing chromatographic peaks. Significant chloride concentrations in the rice matrix can cause an isobaric interference in the ICP-MS due to the $^{40}Ar^{35}Cl^{+}$. If the chloride peak falls directly on top of any arsenic peak with respect to retention time, there may be an overestimation of concentration of that particular arsenic species.

Limits of detection were evaluated by injecting standard solutions of varying concentrations, containing all 4 arsenic compounds, onto the column. The concentrations were the following: 0.1, 0.5, 1, 5 and 10 μg L^{-1}.

3.4 Results and Discussion

3.4.1 Extraction of Arsenic

As can be seen from **Figure 3.4** and **Figure 3.5**, when the rice sample extract solutions are dialyzed, more As(V) is recovered. **Figure 3.4** shows a larger peak area for As(V) in the water extraction procedure, while **Figure 3.5** shows the same effect in the nitric acid extraction procedure. This could be due to the increased extraction time, as the dialysis runs for an additional 3 hours, after the microwave extraction is complete.

Carbon concentrations in each extract solution were also measured. As can be seen from **Table 3.5**, the nitric acid extract solutions contain a large concentration of dissolved carbon, about 4 g L^{-1}, depending on the rice sample In this case, the dilute nitric acid begins to break down the larger amylose and amylopectin starches in the rice extract solution. These smaller molecules can then pass through the pores of the dialysis bag. Nitric acid extractions, therefore, prove difficult to clean-up. The residual carbon concentrations in solution remain high.

In addition to causing an increased carbon concentration in the injection solution, nitric acid extractions also lead to differences with the chromatographic peaks. As shown in **Figure 3.5**, an additional peak appears after the As(V) peak has eluted. This unknown peak does not match up with any of the 4 arsenic species standard peaks investigated in this study. The nitric acid in the sample extract solution may be causing a slight pH change in the chromatographic column. This pH gradient may result in a shift of retention times for one of the arsenic species, or it may lead to a splitting of one of the arsenic species peaks, depending on the pK_a's of each compound and the varying pH in the column. This unknown peak in the chromatogram from the injection of the nitric acid extract should be further investigated and the arsenic species present should identified.

Because of the elevated carbon concentrations in the rice extract solution, and the appearance of an unknown peak in the chromatogram, efforts to speciate arsenic compounds in the rice grain with nitric acid were abandoned. The water extraction procedure is therefore recommended as the better extraction procedure with respect to sample clean-up and interconversion of arsenic species within the separation. The microwave-assisted water extraction procedure, followed by dialysis of the sample extract proved to be an accurate method for the determination of As(III), As(V), DMA and MMA in rice grains.

3.4.2 Sample Clean-up Procedures

Optimization led to a 2 hour dialysis time. As can be seen from **Figure 3.6** and **Figure 3.7**, the optimum dialysis time did not depend on the sample matrix being present.

After 2 hours all of the arsenic species equilibrated in solution across the dialysis membrane. It should be noted that As(III) and DMA species equilibrate almost instantaneously, while both the As(V) and MMA species take about 2 hours to equilibrate. This could be due to the fact that As(III) and DMA are neutral at the solution pH, while the As(V) and MMA species are charged. It appears that the charged species take longer to cross through the dialysis membrane. After 2 hours, all of the 4 arsenic species have equilibrated.

As can be seen in **Table 3.2**, the carbon signal for the dialyzed water extract significantly decreased, from 1,075,077.8 cps to 28,021.8 cps. This confirms that the dialysis procedure was successful in reducing the amount of carbon-containing compounds such as starch in the extract solution. The carbon signal for the nitric acid extracts remained about the same before (1,304,880.7 cps) and after (1,252,162.5 cps) the dialysis. In this case, the nitric acid decomposes the starch molecules into smaller carbon-containing compounds that are able to pass through the pores in the dialysis bag.

3.4.3 Separation and Detection by HPLC-ICP-MS

HPLC parameters were optimized to obtain the best resolution in the shortest amount of time. Parameters were varied with respect to mobile phase pH, flow rate, and injection volume. The viable pH range was ±1 pH unit, and a final elution time greater than 15 minutes was considered suboptimal. The detection capability of the method was examined for adequate signal to noise ratio that was recognized by the Chromera® chromatography software. As can been seen in **Figure 3.8**, the flow rate was varied from

0.8 mL min^{-1} up to 1.2 mL min^{-1}. The elution time of the As(V) peak was reduced by about 90 s as the flow rate increased from 0.8 to 1.2 mL min^{-1}. The resolution of the peaks was not compromised by this increase in flow rate.

For the optimum method, the chlorine peak elutes after the As(V) peak. This is illustrated in **Figure 3.9**. The chloride peak does not overlap with any arsenic-containing chromatographic peaks, and therefore, no interference effects due to $^{40}Ar^{35}Cl^{+}$ were found.

An estimation of the limits of detection can be seen in the chromatogram presented in **Figures 3.10** and **3.11**. **Figure 3.11** is an expanded view of **Figure 3.10**, focusing on the lower end of the calibration. In **Figure 3.11**, it can be seen that the As(III), DMA and MMA peak areas are still distinguishable from one another down to 0.1 µg L^{-1}. The As(V) peak, however, becomes more difficult to integrate with the Chromera software, and the areas of the peaks become indistinguishable from one another below 0.5 µg L^{-1}.

3.5 Validation

The method was validated with spikes (as standard solutions) into the rice matrix prior to the extraction procedure. As can be seen in **Table 3.3**, the spike recoveries varied from 96 – 106 %. **Table 3.4** shows a mass balance of the arsenic species, compared to the results from the total arsenic nitric acid, microwave-assisted digestion procedure. The method precision is illustrated in **Table 3.5**. This table shows the percentage of arsenic species with respect to total arsenic. The standard deviations of the percentages for each the species does not exceed 5%, showing that the analytical method is precise.

3.6 Conclusions

An accurate method, with respect to published literature values for SRM 1568a, has been developed for the speciation of arsenic compounds [As(III), DMA, MMA, and As(V)] in rice grains. The method is validated by standard solution spikes into the sample matrix prior to the extraction procedure. This suggests that there is no interconversion of arsenic species throughout the entire procedure. A mass balance was also computed for a real rice sample. A certified reference material, SRM1568a, was also used for comparison purposes. The precision related to the determination of arsenic species in the SRM material does not exceed 5%.

In order to accurately access the toxicity of arsenic in rice, arsenic species must first be reliably quantified. The toxicity of arsenic compounds varies greatly with the chemical form present. Not only do accurate methods for the determination of total arsenic need to be established, but more importantly reliable methods for the determination of arsenic species should be developed.

As previously discussed in section 1.4.4, Table 1.3 highlights the inconsistencies in results reported for the speciation analysis of arsenic compounds in SRM 1568a. There are no certified reference values for arsenic species in SRM 1568a, and it can be seen from the literature report that highly variable results have been published. For example, DMA concentrations vary from 31.5 to 180 ng g^{-1}. There is clearly a need to produce a reliable method for arsenic speciation in rice grain where the procedure does not alter the chemical species present in the sample, i.e. no interconversion exists between arsenic species.

The degradation of the chromatographic column becomes a concern when high sample throughput is desired. Extending column life can significantly lower the cost related to replacing columns due to degradation or blockage. By injecting a cleaner sample onto the column, not only is the column life extended, but chromatographic improvements are made with respect to increased peak resolution, decreased peak tailing, and limiting shifts in retention time. Over time, a cleaner sample allows for better chromatographic separations, and increased sample throughput.

Dialysis provides for a substantial advancement in the creating a cleaner sample for HPLC analysis. Molecules are separated based on their size; smaller molecules equilibrate through the bag, while larger molecules like starch polymers cannot move through the pores and therefore remain inside the dialysis bag. For the purpose of this work, dialysis bags made from cellulose were used. Future advancements in the field may provide for pore sites with specific functionalities. Compounds could then be separated based on some chemical property, other than simply based on molecular size.

Advancements in arsenic speciation chromatography depend heavily on the stationary phases available. The Sunfire C8 column provides for better symmetry of peaks as well as better resolution between peaks, because of better end-capping technology used to mask residual silanol groups. Today's technology offers greater versatility in the functionality of stationary phases. New columns are bring manufactured that combine reversed phase stationary phases with added ion exchange capabilities. Residual silanol groups can be end-capped with strong and weak anions and cations to provide multimode separations.[68] This will be beneficial to the future of separations science, and these columns should be investigated for the ability to separate arsenic species as they become available.

Table 3.1 HPLC conditions

Column	Sunfire C8 5 µm, 4.6 x 250 mm with guard column
Mobile Phase*	13.0 mM TBAH with malonic acid to pH 5.80, 1.3% MeOH.
Sample loop	100 µL
Flow rate	1.2 mL min^{-1}

*Mobile phase conditions based on previous work by Pan et al.[38]

Table 3.2 Carbon signal intensities of rice extract solutions after dialysis.

Sample	Carbon intensity (cps)
Water extraction	
centrifuge and filter	1,075,077.8
3 h dialysis	23,021.8
blank	7,404.1
Nitric acid extraction	
centrifuge and filter	1,304,880.7
3 h dialysis	1,252,162.5
blank	12,955.9

Table 3.3 Recoveries of 20 µg L^{-1} standard spike solution into a Carolina Rice sample water extract.

Sample	Arsenic species	Recovery
Carolina rice sample	As (III)	106%
Spike before microwave extraction	DMA	97%
	MMA	103%
	As(V)	102%
Carolina rice sample	As (III)	104%
Spike after microwave, before dialysis	DMA	96%
	MMA	101%
	As(V)	97%

Table 3.4 Mass balance of arsenic species, sum of arsenic species, and total arsenic determined experimentally.

Arsenic Species	Arsenic concentration (ng g^{-1})
As(III)	91
DMA	61
MMA	42
As(V)	83
Sum of As species	277
Total As	283

Table 3.5 Percentage of arsenic species per total arsenic in 8 SRM 1568a samples

	Percentage of Species Per Total Arsenic			
	As(III)	DMA	MMA	As(V)
Sample 1	42	29	1	28
Sample 2	44	23	4	29
Sample 3	47	25	1	27
Sample 4	43	26	1	30
Sample 5	44	19	4	32
Sample 6	33	32	8	28
Sample 7	33	34	6	26
Sample 8	40	30	11	18
Average	41	27	4	27
Standard Deviation	5	5	4	4

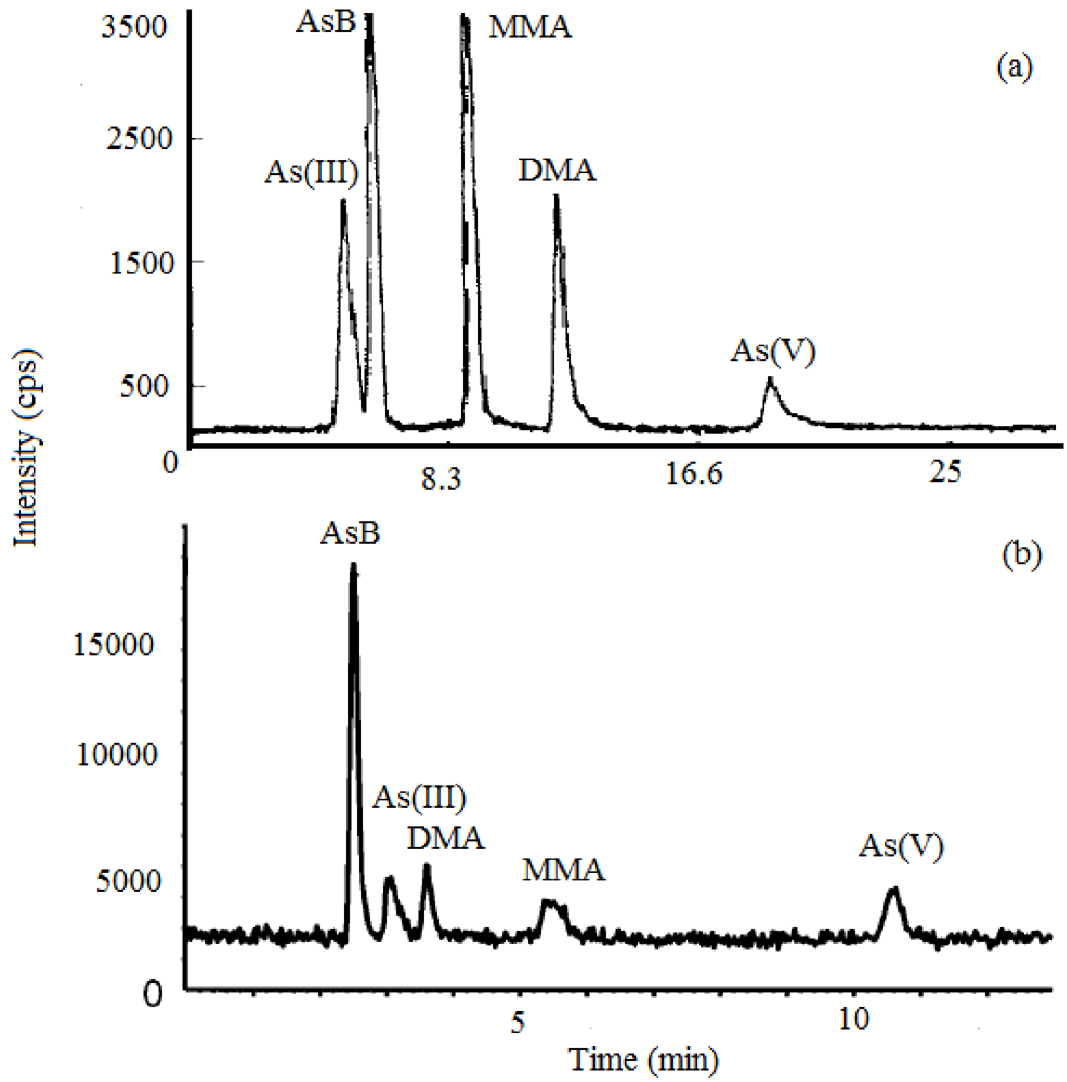


Figure 3.1 Chromatograms of 5 arsenic species separated in human urine samples, showing (a) chromatogram from 1996 and (b) chromatogram from 2012.

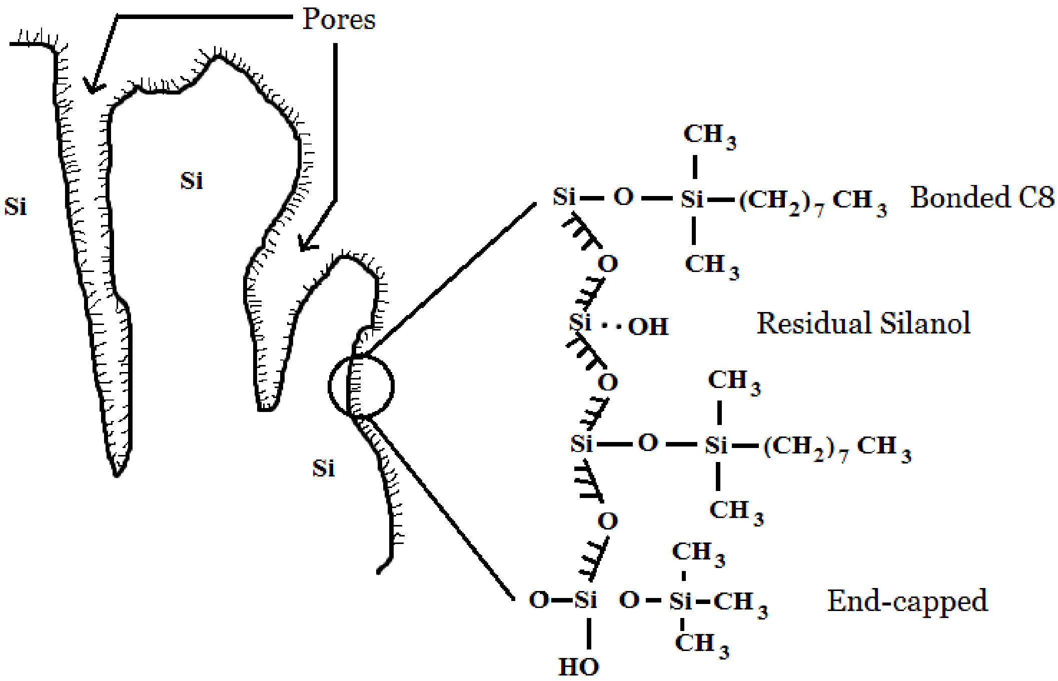


Figure 3.2 Surface structure of a silica-based C8 RPLC column.

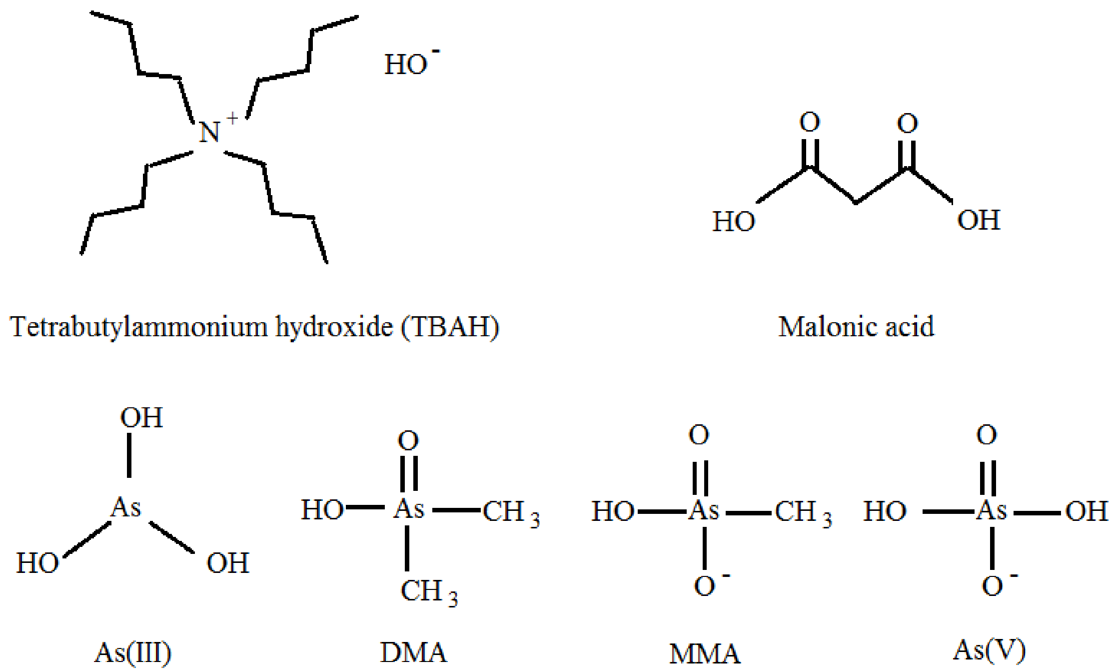


Figure 3.3 Structural representation of TBAH and malonic acid, along with arsenic species present at pH 5.80.

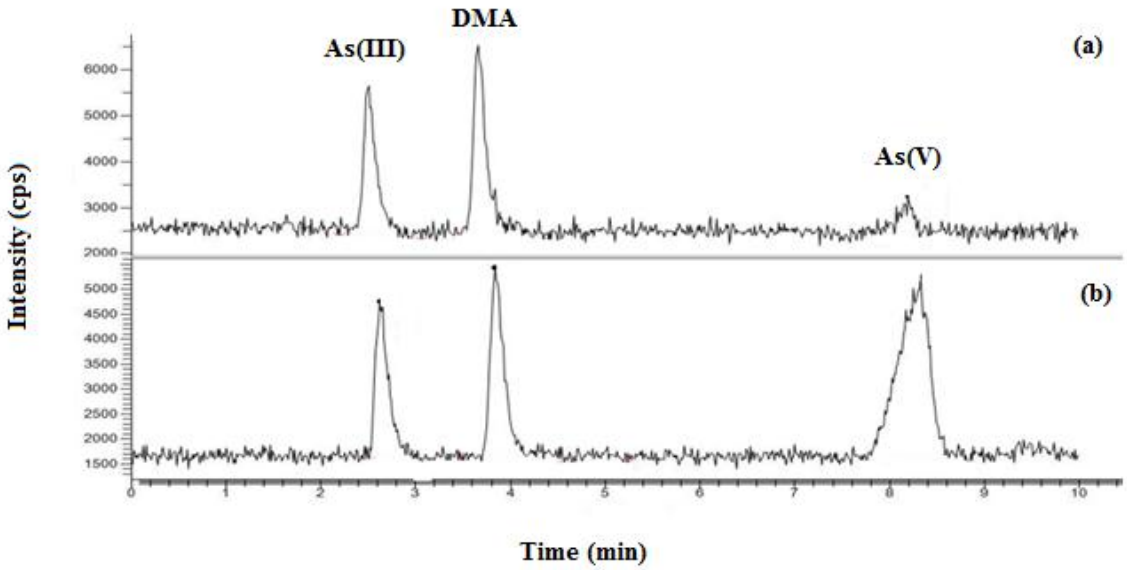


Figure 3.4 Chromatograms of water extraction without (a) and with (b) dialysis

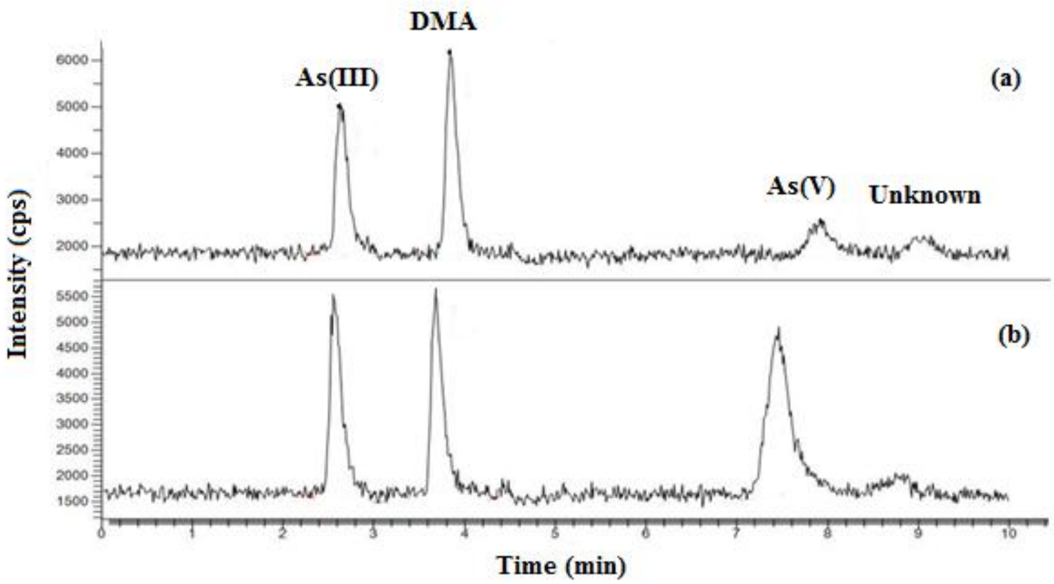


Figure 3.5 Chromatograms for nitric acid extraction without (a) and with (b) Dialysis

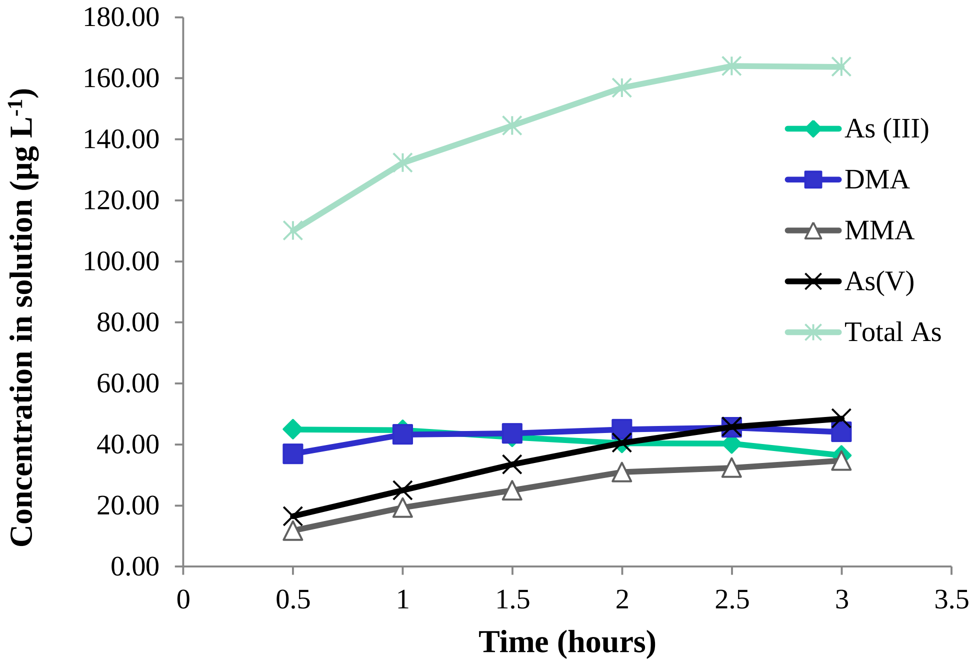


Figure 3.6 Equilibration of dialysis on a standard solution of 24 µg L^{-1} each of As(III), DMA, MMA and As(V).

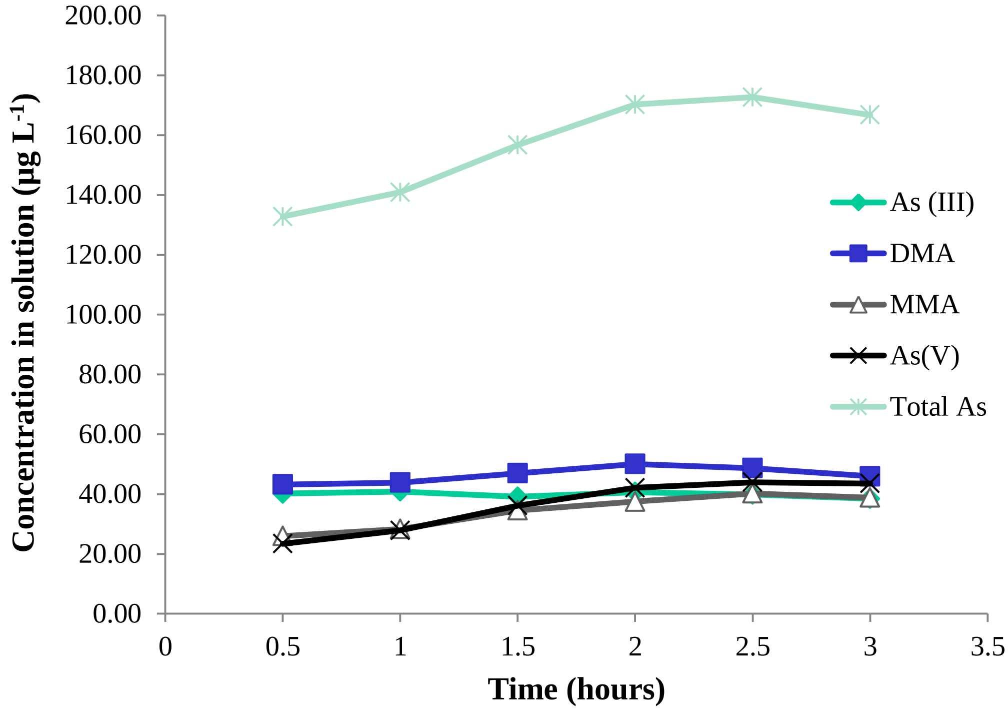


Figure 3.7 Equilibration of dialysis on a standard solution of 24 µg L^{-1} each of As(III), DMA, MMA and As(V) spiked into 0.5003 g rice extract

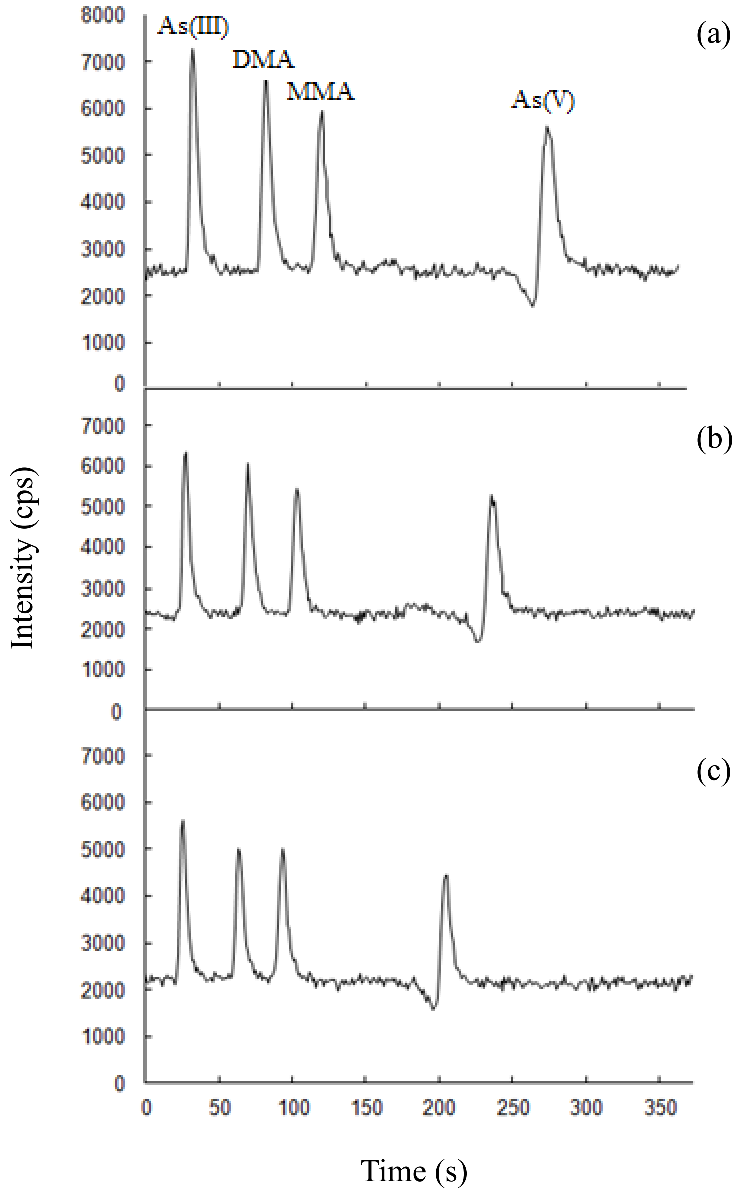


Figure 3.8 HPLC-ICP-MS chromatograms showing separation of As(III), DMA, MMA and As(V), respectively for 0.8 mL min^{-1} (a), 1.0 mL min^{-1} (b), and 1.2 mL min^{-1} (c).

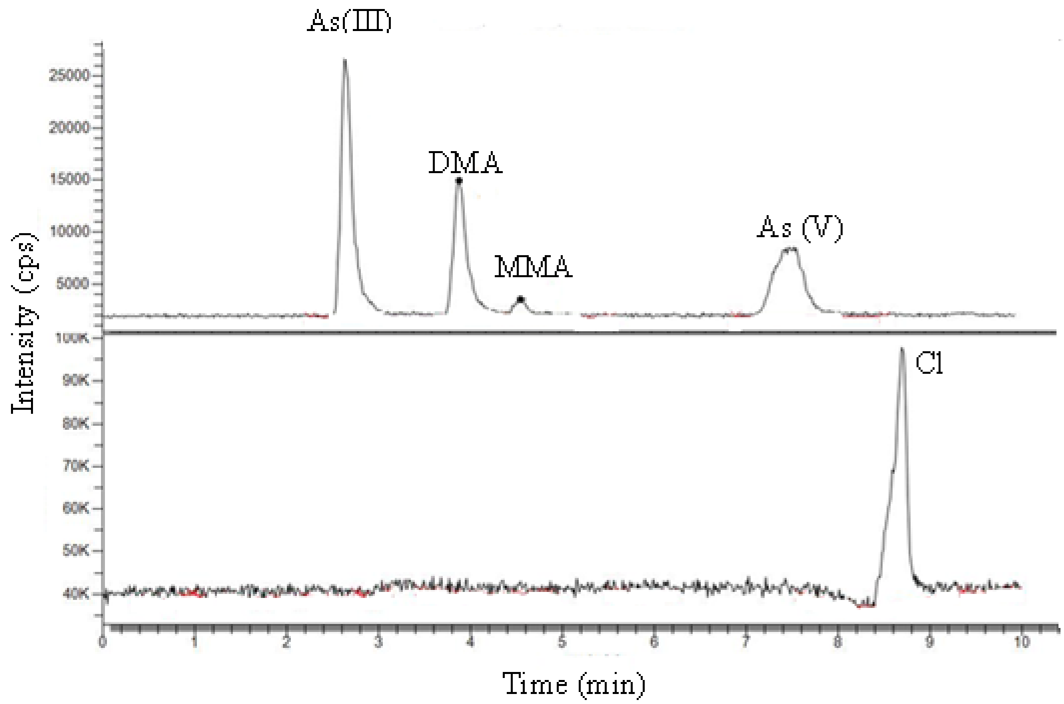


Figure 3.9 Chromatograms of arsenic species and chlorine peak in Carolina rice sample.

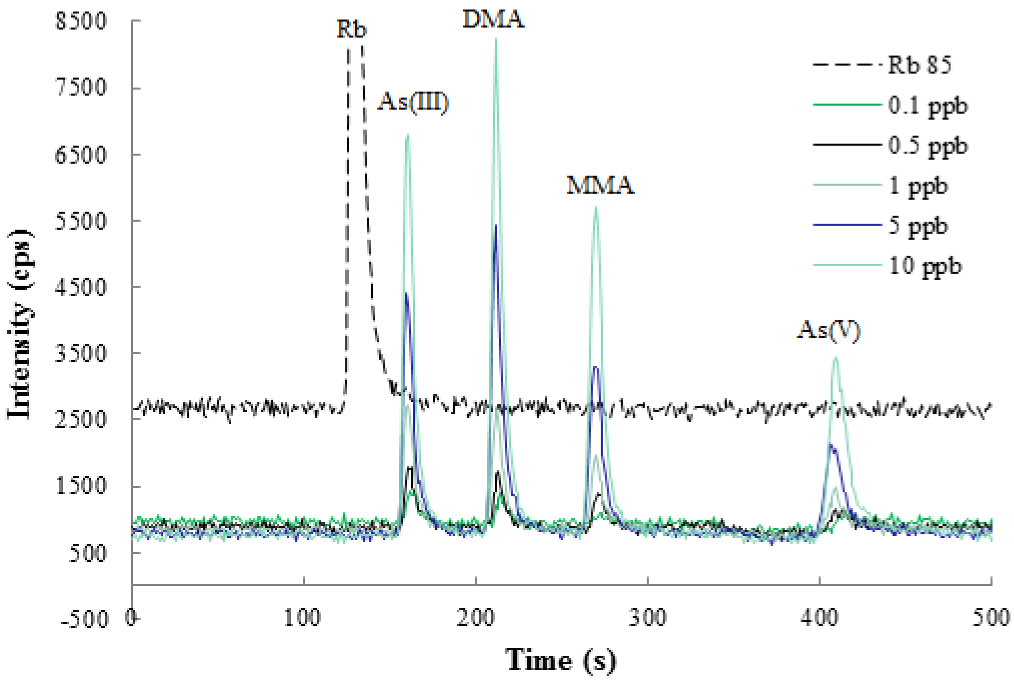


Figure 3.10 Chromatograms of arsenic standard solutions with decreasing concentration.

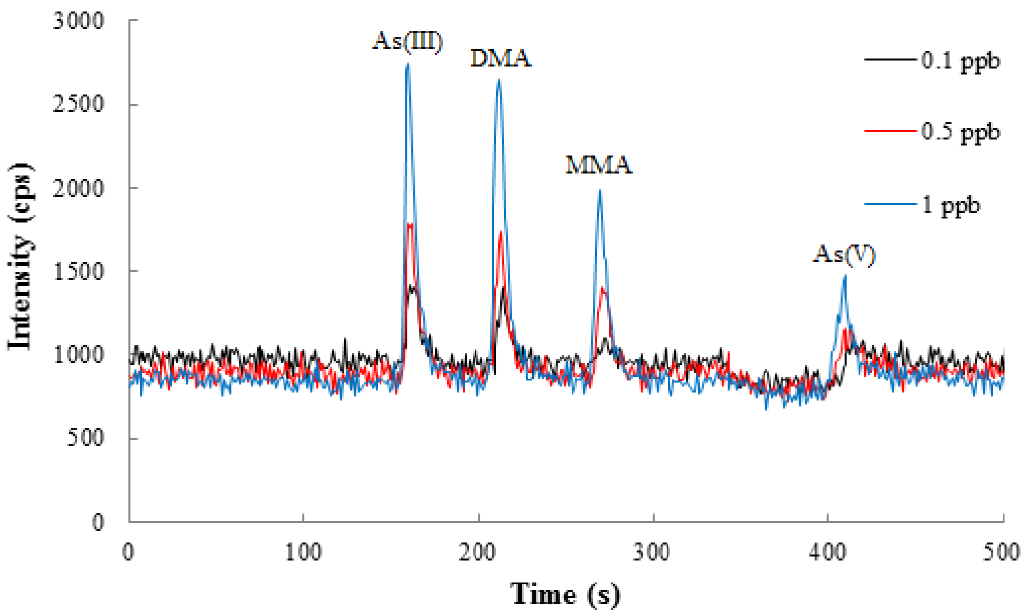


Figure 3.11 Chromatograms of standard solutions of As(III), DMA, MMA and As(V).

CHAPTER 4

IMPROVEMENTS IN REVERSE PHASE-HPLC SEPARATIONS OF ARSENIC SPECIES IN RICE

4.1 Introduction

Analytical chemists are responsible for developing new and improved methods for the reliable identification and quantification of matter. In order to achieve this feat, certain parameters must be established and reported for comparison values. It is often the case with HPLC separations that improvement with respect to chromatographic separations are not carefully examined and produced. In fact, work published in the literature rarely, if ever, report any figures of merit that warrant a significant improvement in HPLC methodology. Parameters such as resolution, peak tailing, and theoretical plate heights are not calculated. It then becomes difficult to distinguish between genuine improvements in the analytical methods versus repeated use of previously reported methods that have not been improved significantly in any manner. To address this issue, it is imperative that future reports of improved HPLC methods include solid evidence of this progress through reports of chromatographic parameters.

The work presented in this chapter provides calculations for figures of merit related to HPLC separations. Comparisons of previously published methods were completed by analyzing chromatograms and calculating resolution, tailing, and theoretical plate heights. Column degradation was also assessed for a Sunfire C8 column over the course of 8 months.

There are several different modes of separation in HPLC, two of which are ion-exchange chromatography and ion-pair reversed phased liquid chromatography (RPLC). In ion-exchange chromatography, the ionic functional groups are chemically bonded to the stationary phase. The ionic group then interacts with analyte ions of opposite charge. In ion-pair RPLC, ion-pairing agents are used in the mobile phase. These ionic compounds usually possess a hydrocarbon chain that helps its retention on the stationary phase.

HPLC separations are often evaluated by several figures of merit including elution time, peak tailing, resolution, and number of theoretical plates. Elution time should be as short as possible without compromising resolution between peaks. For arsenic speciation analysis, elution times below 10 minutes have generally been achieved.

Peak tailing is a measure of a peak's asymmetry, or a peak's deviation from the ideal Gaussian curve. The US Pharmacopeia (USP) defines the tailing factor (T_f) by the following equation:

$$T_f = \frac{a+b}{2a}$$

where a and b and the fronting and tailing half-widths at 5% of the peak height. Peak tailing results from silanol groups present in the stationary phase. According to Dolan,[91] if a silanol group is exposed to adjacent silanol groups, the two can share a proton. The silanol groups are more acidic than the surrounding groups on the stationary phase. Peak tailing occurs because the acidic silanol groups interact more strongly with basic solutes. Therefore, when basic solutes are separated, peak tailing can occur. When trace metal impurities are present in the stationary phase, they can act as ion-exchange sites. When the trace metals are adjacent to free silanol groups, the trace metal can withdraw electrons, causing even more acidic silanol groups. Generally, values for peak tailing should be below 2.

Another figure of merit in the evaluation of chromatographic methods is resolution. Resolution is as measure of how well two peaks are separated and is defined by the following equation:

$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

where A and B are two peaks, t_r is the retention time of the peak, and W is the base width of the peak. Baseline resolved peaks have an R value above 1.5.

The number of theoretical plates can also be calculated. A theoretical plate is defined as a hypothetical zone in which equilibrium is established in the column. The number of theoretical plates can be calculated by the following equation:

$$N = \frac{5.55\ t_R^{\ 2}}{w_{1/2}^2}$$

where t_r is the retention time of the peak, and $W_{1/2}$ is the width at one half of the peak height. Overall, the greater the number of plates translates into more narrow peaks, which makes the chromatography more efficient. The efficiency of a chromatographic method can be examined by calculating and comparing these various figures of merit.

4.2 Research Objective

The literature shows that previously developed arsenic speciation chromatography is in need of improvement. Deficiencies in the chromatographic methods result in peak tailing or asymmetry, lengthy elution times, and poor resolution between peaks. In the work described in this chapter, the performance of a new stationary phase, the Sunfire C_8, was investigated to improve upon these deficiencies. A method was developed based on minimizing of the issue relating to isobaric interferences due to overlapping chloride and arsenic peaks, and improved detection capabilities.

4.3 Experimental

4.3.1 Chromatographic Columns and Instrumentation

A Sunfire C8, 5 µm, 4.6 x 250 mm column, and a Symmetry C8, 5 µm, 4.6 x 150 mm column, from the Waters Corporation (Milford, MA) were evaluated. The HPLC condition can be found in **Table 4.1**. A Perkin Elmer (Shelton, CT) Series 410 LC pump delivered the mobile phase. The sample was injected onto the column by a Rheodyne 7010 injector equipped with a 7012 injector port and injection loops of 20 µL, 50 µL, and 100 µL from IDEX health and science (Oak Harbor, WA). The column was attached directly to an Elan 6100 plasma source mass spectrometer from Perkin Elmer (Shelton, CT) for detection.

4.3.2 Reagents

All solutions were prepared using 18.1 MΩ cm^{-1} water. Standard arsenic solutions and spikes were prepared from sodium arsenate certified and sodium arsenite (meta) from Fisher Scientific (Fair Lawn, NJ), disodium methyl arsonate hexahydrate, purity 97.5% HPLC grade from Chem Services (West Chester, PA), and cacodylic acid, 98% from Sigma (St. Louis, MO). Mobile phase for HPLC separations was prepared from tetrabutylammonium hydroxide, 1.0M solution in methanol from Sigma-Aldrich (St. Louis, MO). The pH of the mobile phase was adjusted with malonic acid, Reagent Plus 99% from Sigma-Aldrich (St. Louis, MO).

4.3.3 Data Processing

The number of theoretical plates, peak resolution and peak tailing were calculated by Chromera software. Analyzing Digital Images (ADI) software was used to evaluate published chromatograms. ADI is available without charge from www.umassk12.net/adi.

4.3.4 Comparison of Chromatographic Columns

Improved chromatographic separations should be assessed with the following performance parameters in mind: total elution time less than 10 min, peak tailing less than 2, and resolution between the DMA and MMA peaks above 1.5. The effect of mobile phase composition was previously reported by Pan, et al.[38], and was therefore not investigated. The separation was completed with isocratic elution, in order to avoid the potential issues that gradient elution causes of the mass spectrometer detector. The chromatography was completed at room temperature in order to be readily adaptable in the laboratory setting, as setting column temperature involves additional equipment. Limits of detection were also calculated based on visual inspection of peaks.

4.3.4.1 Optimization of Flow Rate

The flow rate for each column was optimized. Flow rates varying from 0.8 mL min^{-1} to 1.4 mL min^{-1} were evaluated. Optimal flow rates were decided based on a total elution time less than 10 minutes and peak resolution.

4.3.4.2 Limits of Detection

A series of mixed calibration standard solutions containing all 4 arsenic species [As(III), DMA, MMA and As(V)] were injected onto the column. The standards were made up in water, and ranged from 0.1 to 10 µg L^{-1}. Detection limits were assessed visually by examining peak shapes where the area under the peak is visually distinguishable from the baseline.

4.3.4.3 Investigation of $^{40}Ar^{35}Cl^{+}$ Interference

Sodium chloride was added to standard arsenic solutions. The $^{40}Ar^{35}Cl^{+}$ isobaric interference in ICP-MS detection of arsenic species was monitored. The peaks at m/z 75 and 35 were simultaneously monitored to evaluate the extent of overlap between the arsenate and chloride peaks.

4.3.4.4 Column Life

Chromatograms from different time points were examined for shift in retention time, loss of resolution, increased peak tailing, and changes in peak asymmetry. The column was evaluated over an 8 month period during which 195 standard solutions and samples were injected.

4.3.5 Comparison of Chromatographic Methods

A literature survey was conducted in order to relatively compare current HPLC methods for the speciation analysis of arsenic compounds. Chromatograms were extracted from the PDF files of work published in the literature. Work was selected based on if a chromatogram was present in the file. The image was uploaded to the ADI software. The digital images were examined, and an internal scale was created from the chromatographic axes. Peak heights, peak widths, and elution times were traced with the line tool in the application, which outputs a value based on internal scale. These values were then used to calculate retention times, plate height and number, peak resolution, and peak symmetry.

4.4 Results and Discussion

4.4.1 Optimization of Flow Rate

The optimization of flow rate for the Symmetry C8 column is illustrated in **Figure 4.1.** As the flow rate was increased from 0.8 mL min^{-1} to 1.2 mL min^{-1}, the total elution time decreased from 260 s to 180 s. The optimization of flow rate for the Sunfire C8 column is illustrated in **Figure 4.2.** As the flow rate was increased from 0.8 mL min^{-1} to 1.2 mL min^{-1}, the total elution time decreased from 277 s to 210 s. It should be noted that the Symmetry column is only 150 mm in length, while the Sunfire column is 250 mm in length. Although the Sunfire column is 100 mm longer, the final elution time for the As(V) peak, the last to elute, does not dramatically increase. In fact, the elution times only differ by 20 s. This shows the efficient separation occurring within the Sunfire column.

4.4.2 Limits of Detection

The limits of detection for the arsenic species separated by the Sunfire column can be seen in **Figure 4.3**. Visual inspection of the chromatographic peaks leads to the conclusion that for As(V), the areas under the curve below 1 µg L^{-1} are not distinguishable from one another. The peak tailing makes it difficult for the Chromera calculate accurate baselines, and therefore, peak areas become difficult to interpret.

4.4.3 Investigation of $^{40}Ar^{35}Cl^{+}$ Interference

The $^{40}Ar^{35}Cl^{+}$ isobaric interference in ICP-MS detection of arsenic species was investigated by monitoring m/z 75 and 35 simultaneously. As can be seen in **Figure 4.4**, the chlorine peak overlaps with the As(V) peak at about 200 s. Although the concentrations of chlorine in rice digest solutions does not cause as increase in the arsenic signal response, if samples containing higher concentration of chlorine were analyzed, a problem may arise with the isobaric interference. Ideally, the two peaks should be well separated, so that there is no questionable data related to the effects of chlorine on the arsenic signal response. It is therefore suggested that, under the mobile phase conditions discussed in this chapter, the Symmetry column is not suitable for arsenic speciation analysis when there are concentrations of chlorine in the percentage range in the sample. As previously discussed in Chapter 3, **Figure 3.6** the chlorine peak emerges well after the As(V) peak elutes from the Sunfire column and there is no isobaric interference to affect the accuracy of the arsenic determination.

4.4.4 Column Life

Chromatograms from different time points were examined for shift in retention time, loss of resolution, increased peak tailing, and changes in peak asymmetry. The values can be seen in **Table 4.2**. For the DMA peak, the retention times ranged from 3.228 to 4.743 min, the number of plates varied from 1,968 to 4,663, the resolution ranged from 1.74 to 5.59 min, and the tailing factor varied from 1.756 to 2.439. The data do not support the theory that systematic drift exists.

4.4.5 Comparison of Chromatographic Methods

The results of the comparison of the chromatograms taken from the literature are shown in **Table 4.3**. The tailing factor for the Sunfire column does not exceed 1.2, whereas the values obtained by Batista et al.[73] and Narukawa et al.[72] reach 1.4. This means that the peaks from the Sunfire column are more symmetrical. This could be due to the decreased number of exposed free silanol groups in the stationary phase.

Resolution values exceeding 1.5 are obtained from peaks that are completely baseline separated. Although resolution between the DMA and MMA peaks for the Sunfire column is 1.2, the value is similar to values published in the literature.

4.5 Conclusions

The Sunfire column has promising characteristics for improved chromatographic separation of arsenic species. The Sunfire C8 column has improved end-capping of silanol groups that allow for significant improvements in the separation of arsenic compounds. There is better resolution between peaks, indicating that a more uniform stationary phase has been developed. The pore size of the stationary phase may also have been improved so that when ion-paired with TBAH the arsenic compounds move through the pores more effectively, resulting in better resolution between peaks. Compared to the Symmetry column, the Sunfire column has better resolution between the DMA and MMA peaks and also allows for the complete separation of the chlorine peak from that of the As(V) peak. The values of the chromatographic performance parameters for the

Sunfire column are also similar to the values calculated from reports published in the literature.

The data reported on chromatographic figures of merit are original, and have not been previously disclosed. The literature on HPLC separations is largely silent when it comes to performance parameters with no reports mentioning resolution or tailing calculations. In order to improve upon chromatographic separations, it is recommended that these parameters be reported in the literature.

There is also little information provided concerning the kinetics of HPLC separations. It is not clear whether the carbon chains located on the ion-pairing agent (in this case TBAH) interacts initially with the stationary phase hydrocarbon chains, essentially coating the surface of the stationary phase with ionic charges, which in turn interacts with the analyte. Another possibility is that the ion-pairing agent interacts initially with the analyte ions, exposing the hydrocarbon chains which in turn interact with the stationary phase hydrocarbon chains. Information about the kinetics of this reaction would be useful when determining how to improve upon the chromatographic conditions.

Table 4.1. HPLC conditions.

HPLC Conditions	
Instrumentation	Biosystems 400 Solvent Delivery System
Columns	Waters Symmetry C8 5 µm 4.6 x 150 mm
	Waters Sunfire C8 5 µm 4.6 x 250 mm
Mobile Phase*	13.0 mM tetrabutylammonium hydroxide, 1.3% methanol pH 5.80 adjusted with malonic acid
Injection Loop	20 µL
Void Volume Marker	Rb plasma standard solution
Arsenic Species Concentration	10 µg L^{-1} as As of sodium arsenite, dimethylarsinic acid, disodium methylarsonate hexahydrate, and sodium arsenate

*M.P. conditions from Pan et al.[38]

Table 4.2 Number of plates, resolution and tailing factors for injections of a 10 µg L^{-1} each of As(III), DMA, MMA and As(V) at various times between July 12, 2011 and February 28, 2012.

Date	Injections	Peak	Retention Time	Number of Plates	Resolution	Tailing Factor
07.12.2011	43	As(III)	2.287	2,728		2.709
		DMA	3.228	4,663	5.17	1.952
		MMA	3.974	4,926	3.59	1.787
		As(V)	6.730	5,464	9.33	1.354
07.20.2011	49	As(III)	2.438	2,804		2.946
		DMA	3.446	4,621	5.21	2.186
		MMA	4.213	5,358	3.54	1.704
		As(V)	7.047	2,491	7.13	1.700
08.17.2011	60	As(III)	2.583	1,577		1.854
		DMA	3.672	3,530	4.29	1.756
		MMA	4.321	4,608	2.59	1.641
		As(V)	7.884	27,258	15.99	0.768
09.14.2011	76	As(III)	3.403	2,775		2.256
		DMA	4.743	3,517	4.63	1.929
		MMA	5.825	5,215	3.37	1.419
		As(V)	9.353	2,803	6.85	1.450
09.15.2011	83	As(III)	3.154	1,984		2.385
		DMA	4.418	4,399	4.60	2.439
		MMA	5.431	5,603	3.64	1.606
		As(V)	8.672	2,776	6.83	1.522
10.08.2011	97	As(III)	2.644	1,592		2.165
		DMA	3.872	3,540	1.74	2.013
		MMA	4.581	3,883	2.56	
		As(V)	8.500	6,188	10.79	1.088
11.29.2011	169	As(III)	2.728	1,948		2.062
		DMA	4.174	3,805	5.59	1.707
		MMA	4.714	4,602	1.97	1.281
		As(V)	9.171	13,104	14.90	1.292
12.14.2011	180	As(III)	2.550	1,093		1.995
		DMA	3.814	1,968	3.88	1.944
		MMA	4.495	2,533	1.94	1.679
		As(V)	7.923	1,201	0.60	1.256
02.28.2012	195	As(III)	2.671	2,040		2.049
		DMA	3.908	3,502	4.94	2.028
		MMA	4.573	5,616	2.62	1.552
		As(V)	7.533	2,438	6.93	1.098

Table 4.3 Literature values for theoretical plates, tailing factors, height equivalent to a theoretical plate (HETP), and resolution.

Column	Peak	Theoretical Plates (N)	Tailing Factor (T_f)	HETP (mm)	Resolution	Reference
Sunfire C8, 5µm, 250 mm x 4.6 mm	As(III)	2118	1.2	0.118		This study
	DMA	3016	1.1	0.083	2.0	
	MMA	3485	0.6	0.072	1.2	
	As(V)	3167	1.0	0.079	4.6	
PRP-X100	As(III)	1693	1.3	n.d.		[73]
	DMA	3969	1.4	n.d.	1.1	
	MMA	8457	1.1	n.d.	1.4	
	As(V)	6598	1.2	n.d.	8.3	
ODS L-column, 150 mm × 4.6 mm	As(V)	8034	1.3	0.019		[43]
	As(III)	12446	1.4	0.012	2.4	
	MMAA	6542	1.2	0.023	1.2	
	DMA	14305	1.3	0.010	2.1	
PRP-X100, 250 mm x 4.6 mm	As(III)	1921	1.1	0.130		[68]
	DMA	4378	1.1	0.057	1.6	
	MMA	7188	0.9	0.035	3.6	
	As(V)	6915	1.1	0.036	3.3	

n.d. means the values were not determined because column de=imensions were not reported in the literature.

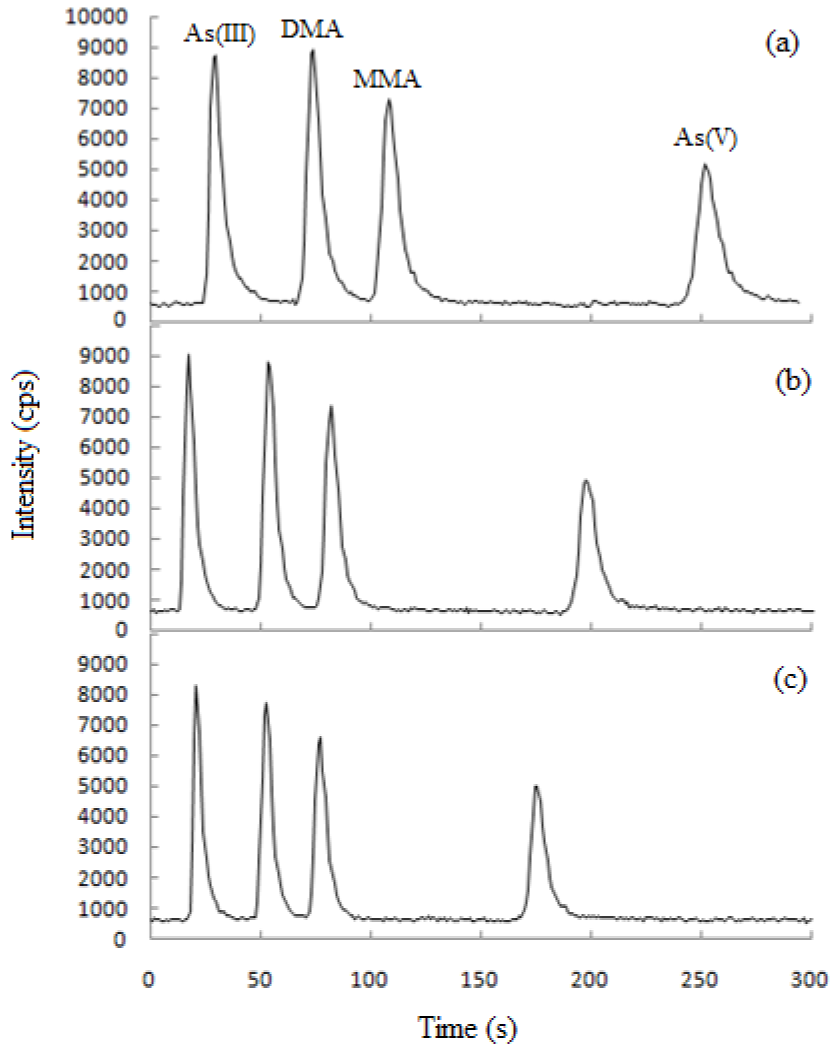


Figure 4.1 HPLC-ICP-MS chromatograms showing separation of As(III), DMA, MMA and As(V), respectively for 0.8 mL min^{-1} (a), 1.0 mL min^{-1} (b), and 1.2 mL min^{-1} (c) on the Symmetry C8 column.

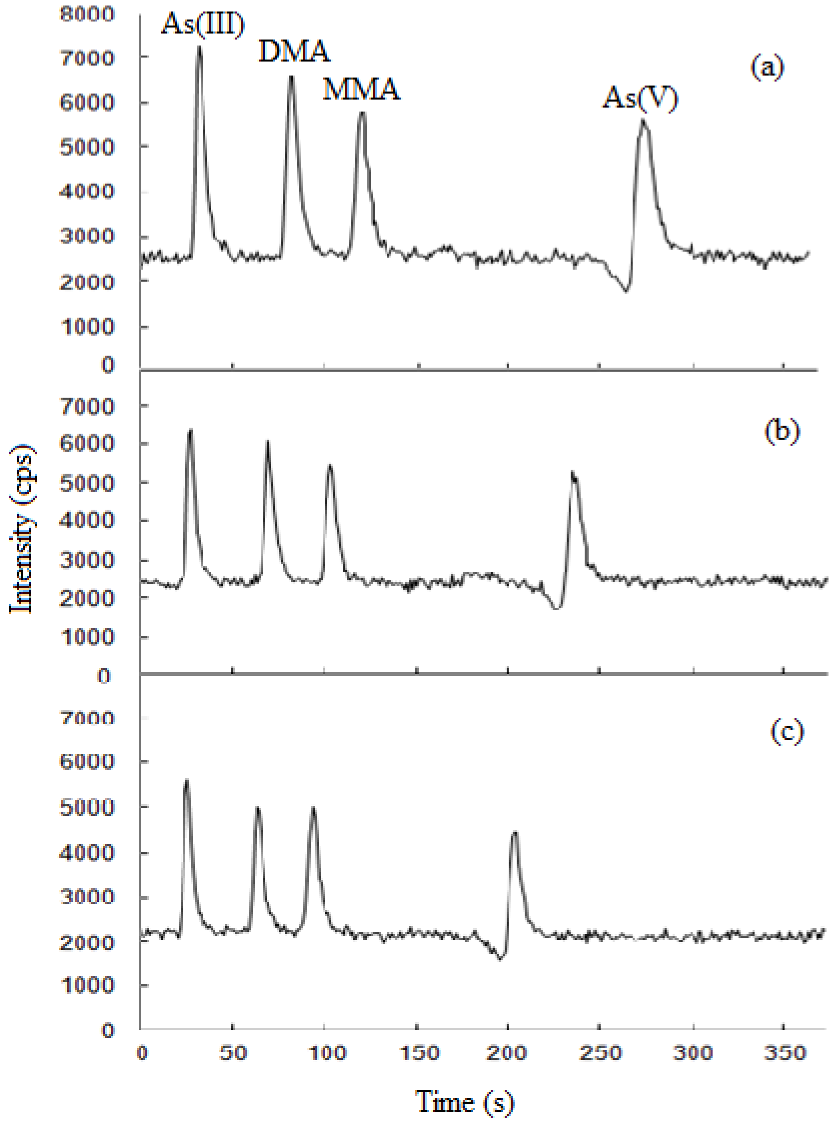


Figure 4.2 HPLC-ICP-MS chromatograms showing separation of As(III), DMA, MMA and As(V), respectively for 0.8 mL min^{-1} (a), 1.0 mL min^{-1} (b), and 1.2 mL min^{-1} (c) on the Sunfire C8 column.

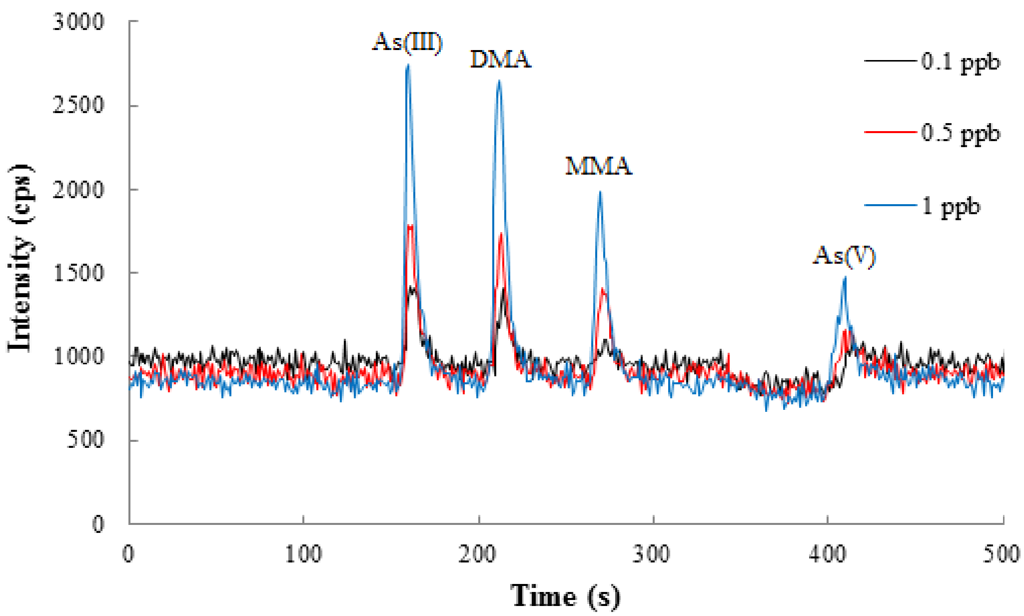


Figure 4.3 Chromatograms of standard solutions of As(III), DMA, MMA and As(V) ranging from 0.1 to 1 μg L^{-1} for the Sunfire column.

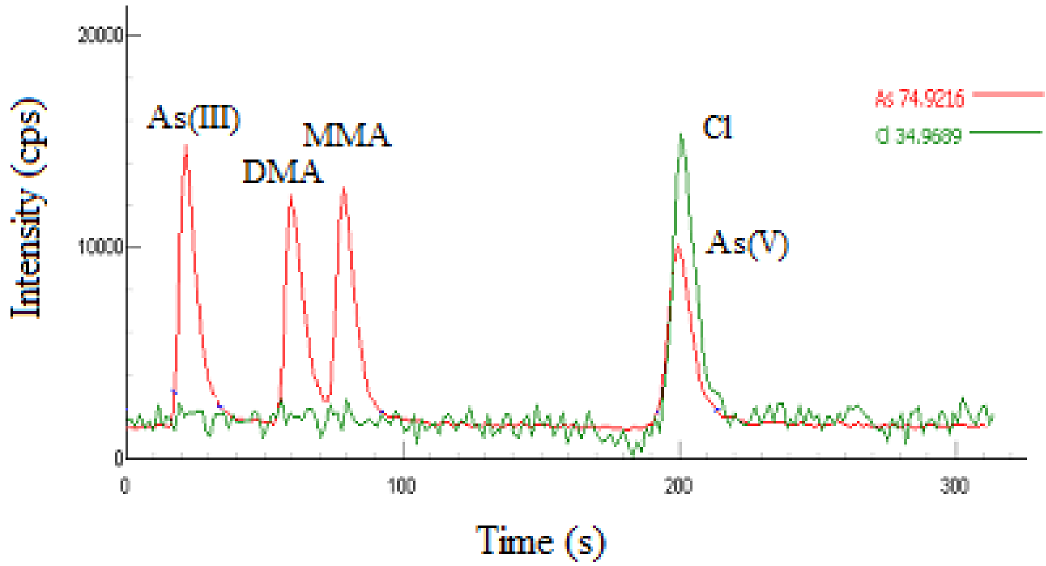


Figure 4.4 Chromatogram showing overlapping spectra of As(V) peak with chloride peak on the Symmetry column. (Cl trace is vertically shifted for comparison purposes.)

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

An accurate and precise method was developed for the quantification of total arsenic in rice grain material. The experimental design was based on the assumption that the response of the instrument was not affected by the digestion parameters. However, the following affects were recognized:

- the amount of residual dissolved carbon may affect the extent of ionization of arsenic in the plasma and hence the sensitivity.
- chlorine-containing reagents (such as hydrochloric acid) were not a good choice because of the $^{40}Ar^{35}Cl^{+}$ isobaric interference with arsenic at m/z 75.
- effects related to acid concentration must also be accounted for.

The digestion parameters investigated were particle size, sample mass, and various combinations of reagents, temperature programs, and venting procedures. The method was validated by measuring recovery of arsenate spikes and the analysis of a certified reference material.

Methods for speciation analysis for arsenate, arsenite, DMA and MMA in rice grain material were successfully performed. Microwave extraction procedures with water are sufficient to extract arsenic from rice grains. Dialysis of the sample extract served to "clean-up" the final solution that was injected onto the column. This will prolong column life by decreasing the amount of large starch molecules injected onto the column. The method was validated by measuring recovery of spikes into the rice matrix and by comparing literature values for speciation information with regards to the analysis of certified reference material, SRM 1568a rice flour.

The Sunfire C8 column is superior to the Symmetry C8 column, in that there is better resolution between the peaks for DMA and MMA, and less peak tailing. The performance of the Sunfire column does not show any degradation thus far more details. Also, the chloride peak elution from the Sunfire column occurs after all 4 arsenic species have eluted. When the Symmetry column is used, the chloride peak lies directly on top of the As (V) peak.

5.2 Future Work

Accurate speciation methods for the determination of arsenic in rice need to be developed further in order to establish reliable toxicity studies as well as in attempting to produce national or global recommendations and regulations for the arsenic content of rice. Arsenic in rice is a significant global issue that, in coming years, will need to be addressed by governing legislative bodies. In order to affectively establish sound regulations for arsenic concentrations in foodstuffs effectively, particularly in rice, there is a need for reliable speciation methods.

Recently, the difficulty in providing sound analytical methodology was highlighted in the results from a proficiency test set up for the determination of total arsenic and inorganic arsenic in rice. In the IMEP-107 study,[33] researchers attempted to homogenize a rice sample as part of a study in which the bulk rice material was cryogenically milled to a particle size below 250 µm and homogenized for 30 min. Bottles containing 20 g of powered material were then delivered to the participating laboratories. According to the original lab, the samples were both homogeneous for total and inorganic arsenic, and stable for over 6 weeks. Reference values for total and inorganic arsenic content was established by 7 expert laboratories. The sample was then sent out to 103 separate laboratories from 35 different countries. Of the labs that participated in the study, 75% performed satisfactorily, and only 60% provided realistic uncertainty estimates. Moisture content in the samples varied also from 0.5 – 14 %. From this information, the facilitators of the proficiency test stated that there was no reason not to consider introducing maximum levels for inorganic arsenic for further discussions on risk management. It can be seen, however, that there was a wide range of results, with a high percentage of the laboratories scoring outside of the acceptable range. Also, it should be kept in mind that this sample was homogenized and stable. This study highlights the difficulty of accurately measuring arsenic content in rice. If such widely variable results occur from a potential reference material, what can be expected of the analysis of real rice samples? Clearly, there is a need to develop more accurate methods for the determination of arsenic in rice.

The development of these methods requires particular attention being paid to homogeneity of the samples, both between single rice grains as well as within a single bag of rice. This may pose significant issues when setting regulations for arsenic content in rice, as preliminary studies show that rice samples are quite heterogeneous in nature. This is due to the fact that arsenic concentrations can differ significantly from plant-to-plant as well as within a rice paddy. Norton et al.[92] found that there was a 3-34 fold range arsenic concentration in rice grains, in which rice subpopulations were varied. The researchers found correlations between arsenic content in the rice grain and the following factors: genetic variation, year, location and flooding management. This indicated that both breeding strategies and environmental factors could serve to lower arsenic concentration in rice grain. After sampling issues have been dealt with, with respect to heterogeneity of the rice, method development must be further assessed. There is a need to identify and quantify arsenic species lost to drying procedures in the sample preparation steps. There is also a need to develop methods that investigate the larger, less stable arsenic complexes. Both of these methods will probably require preconcentration steps, due to the compounds being present at very low concentrations.

5.2.1 Homogeneity Studies

Heterogeneity of the sample can cause substantial error when reporting results. In order to address this problem, homogeneity of arsenic in individual rice grains and within one commercially available bag of rice should be assessed. Hydride generation – atomic absorption spectrometry would be a suitable technique to evaluate arsenic content within single rice grains. Sun et al.[93] analyzed dietary supplements for inorganic arsenic content by slurry sampling hydride generation atomic absorption spectrometry. Slurries were prepared in dilute hydrochloric acid, employing 8-hydroxyquinolone enhancement effects. The detection limits, calculated as 3s, were 4.0 μg kg^{-1} for As(III) and 4.5 μg kg^{-1} for As(V). The detection limits could provide for an experimental design where individual rice grains are placed directly into the graphite furnace atomizer for AAS detection.

5.2.2 Loss of Arsenic Due to Drying

In order to confirm the identity of the volatile arsenic species lost during oven-drying procedures, GC-MS could be performed on the compound, following cryogenic trapping. This molecular technique would provide vital information about the identity of the unknown arsenic species. The identification of several arsines could be recognized including arsine (molecular weight 77.95 g mol^{-1}, boiling point -55 ºC), monomethyl arsine (molecular weight 91.97 g mol^{-1}, boiling point 2 ºC), dimethylarsine (molecular weight 106.00 g mol^{-1}, boiling point 36 ºC), and trimethylarsine (molecular weight 120.03 g mol^{-1}, boiling point 52 ºC).[82] Trimethylarsine (TMA) is the most probable arsenic species present, due to the reduction and methylation of dimethylarsinic acid present in the rice matrix. Trimethylarsineoxide (TMAO, boiling point 107 ºC) is also a possible intermediate of the reduction/methylation scheme illustrated in **Figure 2.1**. Rice plants are known to detoxify arsenic compounds by complexation with phytochelatin polypeptides.[13] These phytochelatin complexes bind to arsenic through thiol groups, and can also create a reducing environment around the arsenic.

Yuan et al.[94] conducted experiments that confirmed the production of TMA by bacteria expressing the As(III) S-adenosylmethionine methyltransferase gene. TMA accounted for 11-13% of the total arsenic in the sample. Traces of arsine, monomethylarsine and dimethylarsine were also found in the headspace of bacteria samples incubated with As(III), even though there is no known pathway in the bacteria that would be responsible for the formation of these volatile arsines. Combined, the compounds only made up <0.005% of the total arsenic. . Arsine, monomethylarsine and dimethylarsine are therefore not likely to be found as volatile products during the oven-drying procedures for rice. Therefore, studies to identify the volatile arsenic species loss during oven drying should focus on the more likely compounds, TMA and TMAO, that were found in larger quantities in the previously mentioned studies.

Once the unknown volatilizable compounds lost during the oven-drying procedures have been elucidated, further work should be completed to ensure that no degradation of the arsenic species present in rice grains occurs during this sample preparation step. Some alternative methods for the determination of volatile arsenic species are highlighted below.

In the 2011 Atomic Spectroscopy Update[95], the determination of volatile arsenic species released from lake sediments was highlighted. In a study by Yuan et al.,[96] arsine and methylated arsine compounds were separated and concentrated with a short packed cotton column and identified by AFS detection. Limits of detection for AsH_3, CH_3AsH_2, $(CH_3)_2AsH$ and $(CH_3)_3As$ were 2.5, 3.0, 6.5 and 11 pg, respectively. Rice samples could be analyzed in a similar manner, to determine if the volatile species lost during oven drying procedures were arsine compounds.

Biovolatilization of arsenic as methylated arsines is a widespread phenomenon, according to Jakob and Feldmann et al.[97] The stability of MMA, DMA, TMA, and TMAO was studied in atmospheric aerosols by HPLC-ICP-MS/ES-MS. Li et al.,[98] developed a method to separate and identify As(III), As(V), DMA, MMA, and two sulfur-containing arsenic species, dimethyldithioarsinic acid (DMDTA) and dimethylmonothioarsinic acid (DMMTA) in landfill leachates. The researchers accomplished this feat with parallel analysis of the samples by HPLC-DRC-ICP-MS and LC-ESI-MS/MS. A Thermo HyPURITY end-capped C18 column with a mobile phase of 5mM formic acid at pH 2.9 was used to separate the thiol-organoarsenic compounds. This methodology could be applied to rice samples, in order to determine if volatile arsenic species released from the matrix during oven drying can be identified and quantified.

5.2.3 Preconcentration methods

In order to detect trace amounts of larger arsenic complexes that may be present in the rice matrix, improvement in detection capability on the analytical method must be addressed. To detect these species, methods for the preconcentration of the analytes of interest must be investigated. Zheng and Hu[99] provide a separation and preconcentration method for As(III), As(V), DMA and MMA in human hair by employing a dual-column capillary microextraction (CME) system consisting of N-(2-aminoethyl)-3-aminopropyltrimethoxysilane(AAPTS)-silica coated capillary (C1) and 3-mercaptopropyl trimethoxysilane (MPTS)-silica coated capillary (C2). Quantification of the species resulted from detection by electrothermal vaporization-ICP-MS. By incorporating valve switching, As(V)/MMA(V) retained on C1 and As(III) retained on C2 could be sequentially desorbed with dilute nitric acid or thiourea. The concentatration of DMA in the human hair extracts was obtained by subtraction from the total concentration of As in the human hair extracts the sum of the other three As species. Under the optimized conditions, the detection limits (3 s) of the method were 3.9 pg ml^{-1} for As(III), 2.7 pg ml^{-1} for As(V), 2.6 pg ml^{-1} for MMA(V) and 124 pg ml^{-1} for total As. The enrichment factors were 286, 262 and 260 for As(III), As(V) and MMA, respectively.

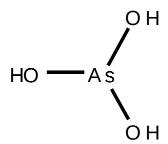
Monasterio and Wuilloud[100] developed a microextraction method for the determination of As(III), As(V) and organic arsenic (DMA and MMA) in water samples. Preconcentration and determination of the species was accomplished using the ion-pairing reagent of tetradecyl(trihexyl)phosphonium chloride ionic liquid (CYPHOS® IL 101). As(V) was selectively separated by forming As(V)-molybdate heteropoly acid [As(V)-MHPA] complex with molybdenum, followed by ion-pairing reaction with CYPHOS® IL 101 and microextraction in chloroform. Arsenic detection was performed by ET-AAS. The extraction efficiency was 99% and the preconcentration factor was 125. The detection limit was 0.002 mg L^{-1} as As(V).

5.2.4 Alternative separation techniques

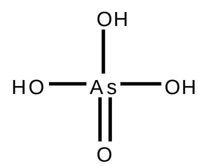
Along with studies into appropriate preconcentration techniques, alternate chromatographic methodology should also be explored. Hydrophilic interaction chromatography has recently been applied to the separation of standard solutions of 9 arsenic species, including phenyl arsine oxide, phenylarsonic acid, TMAO, AsC, AsB, p-ASA, MMA, DMA, and Roxarsone. Xie et al.[101] examined the retention behavior of these organoarsenicals based on Zwitterionic hydrophylic interaction chromatography (ZIC®-HILIC). The researchers determined that retention on the column was based on both hydrophilic partitioning and adsorption driven by hydrogen-bonds with surface h-donor/acceptor groups of the stationary phase. Electrostatic interactions also occurred during the separation. Although the exact mechanism of separation with HILIC is not well known at this time, this study shows some promising results, as applied to arsenic speciation. Further investigation of HILIC separations is, therefore, recommended.

APPENDIX

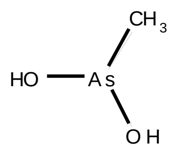
CHEMICAL FORMULAS OF COMMON ARSENIC SPECIES IN ENVIRONMENTAL AND BIOLOGICAL SYSTEMS[12]



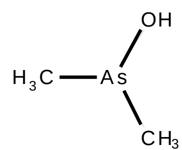
Arsenous acid



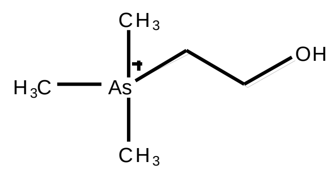
Arsenic acid



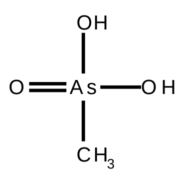
Methylarsonous acid



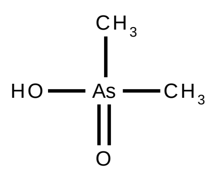
Dimethylarsinous acid



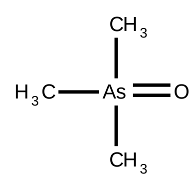
Arsenocholine (AC)



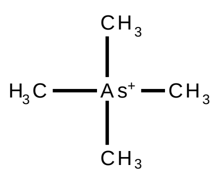
Monomethylarsonic acid (MMA)



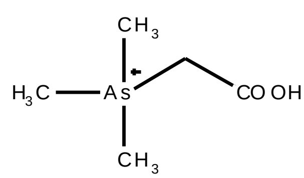
Dimethylarsinic acid (DMA)



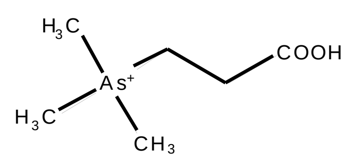
Trimethylarsine oxide (TMAO)



Tetramethylarsonium cation (TETRA)

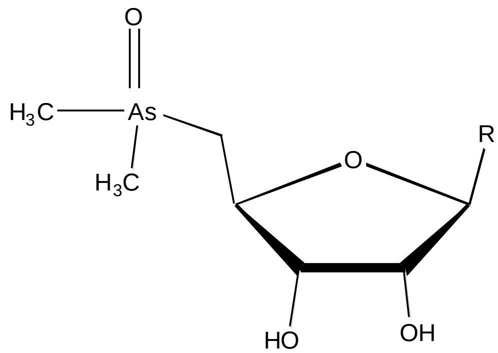


Arsenobetaine (AB)



Trimethylarsoniopropionate

CHEMICAL FORMULAS OF COMMON ARSENOSUGARS SPECIES IN ENVIRONMENTAL AND BIOLOGICAL SYSTEMS



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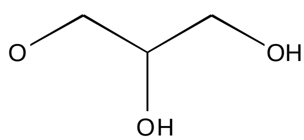
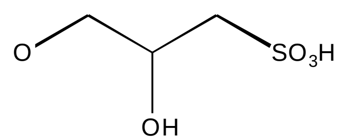
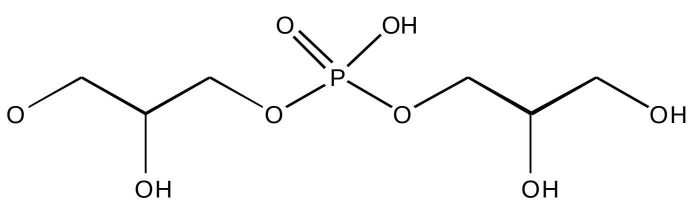


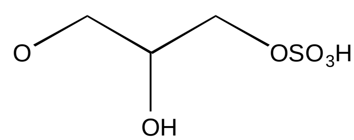
Arsenosugar (Glycerol-ribose)



Arsenosugar (Sulfonate-ribose)

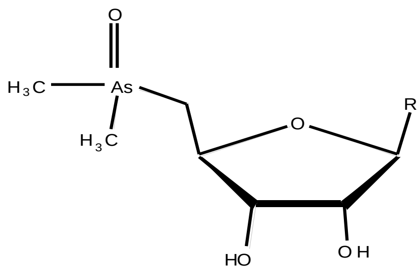


Arsenosugar (Phosphate-ribose)



Arsenosugar (Sulfate-ribose)

CHEMICAL FORMULAS OF SOME WATER-SOLUBLE AND LIPID-SOLUBLE ARSENOSUGAR SPECIES IN MARINE BIOLOGICAL SYSTEMS



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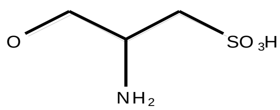

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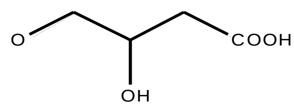

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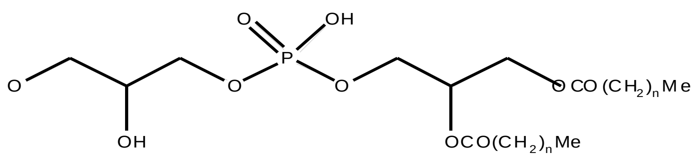

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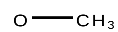

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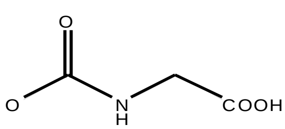

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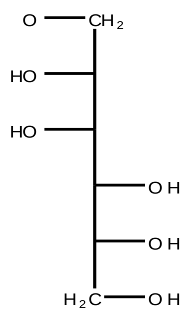

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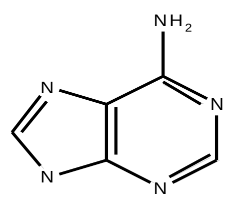

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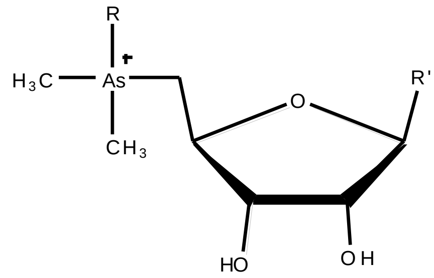
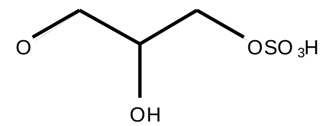
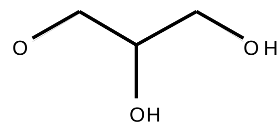
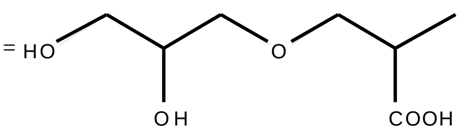
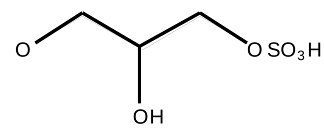
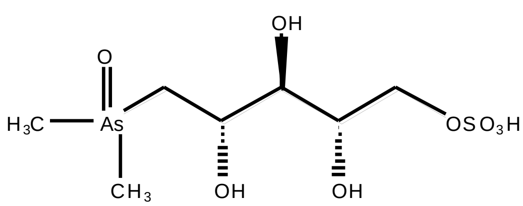


1 R = CH_3 R' = 

2 R = CH_3 R' = 

3 R =  R' = 



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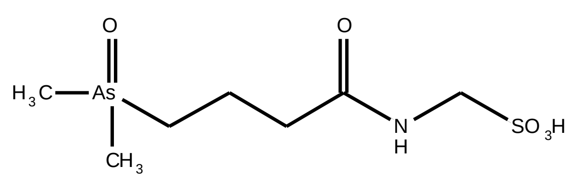

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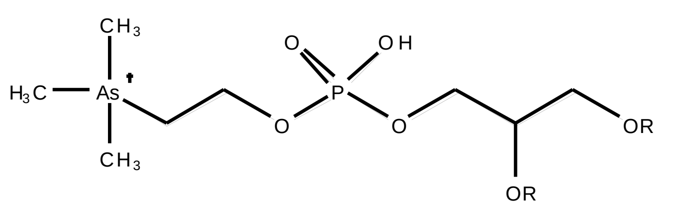

6 R = H **7** R = $CO(CH_2)nCH_3$

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